Using alternate indicators to define need for public health intervention for trachoma: Evidence from the Pacific Islands

ROBERT M. R. BUTCHER

Department of Clinical Research
Faculty of Infectious and Tropical Diseases
LONDON SCHOOL OF HYGIENE & TROPICAL MEDICINE

Thesis submitted in accordance with the requirements for the degree of
Doctor of Philosophy
University of London

Co-funded by the
Fred Hollows Foundation and the **Wellcome Trust**

September 2017

I. ABSTRACT

Introduction: Trachoma is the most common infectious cause of blindness worldwide. The presentation of trachoma in the Pacific small island states varies. This study focuses on Fiji, where the trichiasis prevalence recorded prior to this study was very high, and the Solomon Islands, where the prevalence of trachomatous inflammation – follicular (TF) is high enough to warrant intervention with mass antibiotic treatment, but there is apparently little or no trachomatous trichiasis (TT). This study aims to supplement clinical data with photographic and molecular tools to better characterise presentation and microbiological correlates of disease.

Methods: Pre-intervention population-based prevalence surveys for trachoma were carried out independently and in conjunction with the Global Trachoma Mapping Project (GTMP). Additionally, one focused post-intervention survey was performed. Standardised clinical data collection was supplemented with ocular swab, dried blood spot and photograph collection. Quantitative and sequence-based nucleic acid techniques were used for targeted and non-targeted pathogen detection and characterisation. Enzyme immunoassays were used for serological analysis. Clinical data was supplemented with photographs.

Results: Within the mosaic pattern of clinical trachoma in the Pacific, the prevalence of TT was found to be very low in Fiji and the Solomon Islands. Prevalence of ocular *Chlamydia trachomatis* (*Ct*) infection in these countries was also very low. Further investigations in the Solomon Islands demonstrated *Ct* isolates found to be most closely related to ocular reference strains. Several pathogens that are known to cause follicular conjunctivitis were found, but neither frequency nor load of infection was associated with TF. Amplification of 16S ribosomal RNA amplicons showed diverse ocular microbial communities but no dominant metagenomic communities associated with TF. There is evidence of accumulation of mild scarring as age increases, but little evidence of severe scarring, or association between any trachoma phenotype and exposure to *Ct.*

Conclusion: In Solomon Island communities studied, no evidence was found of significant burden of *Ct* infection, *Ct* transmission, trachomatous inflammation – intense, accumulation of severe scarring in older people or TT. We therefore suspect TF in the Solomon Islands to be of an as-yet unidentified aetiology. The WHO simplified grading system also appeared to lack diagnostic accuracy in Fiji. There are direct implications for implementation of control measures in the Pacific. There are additional connotations worldwide; as the global elimination effort continues and phenotypically similar conditions are unmasked, we suspect the positive predictive value of simplified clinical grading to drop. Use of molecular tools could differentiate communities with a high burden of infection, a key correlate of blinding disease, from those where resources may be better allocated elsewhere.

II. DECLARATION

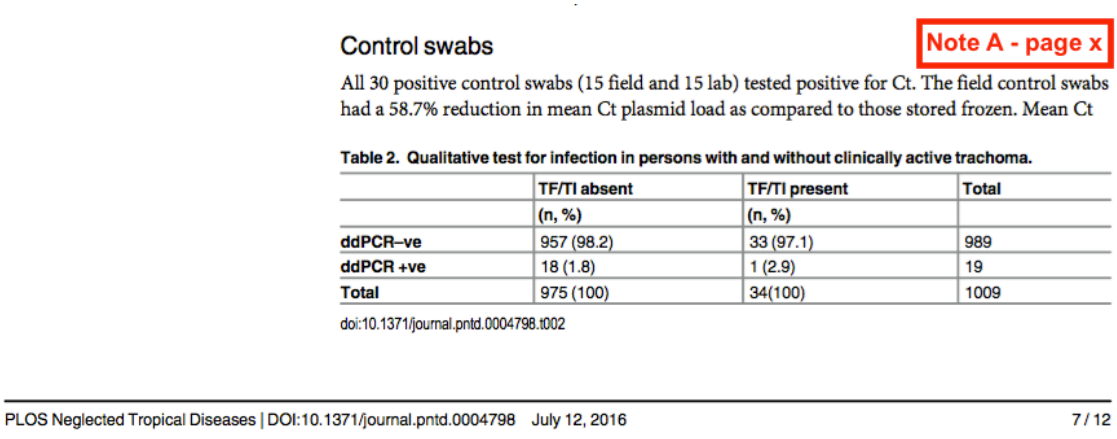
I, Robert Butcher, confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis.

Robert Butcher, September 2017

III. THESIS OUTLINE

In accordance with London School of Hygiene & Tropical Medicine (LSHTM) thesis style guidance, this thesis is presented as a publication-style thesis framed around four key publications in which the author has had a leading role. Prior to each publication, the author's involvement is specified, and the contributions of other authors is acknowledged.

Some of the work that was carried out in pursuance of this degree was not reported in the final papers but is presented in this thesis in the form of supporting notes, information and results. The red boxes marking up the manuscript texts point towards supporting information, for instance the note box in the following example indicates that the reader should read note A on page x for supplementary supporting information on the topic of control swabs.



Control swabs

Note A - page x

All 30 positive control swabs (15 field and 15 lab) tested positive for Ct. The field control swabs had a 58.7% reduction in mean Ct plasmid load as compared to those stored frozen. Mean Ct

Table 2. Qualitative test for infection in persons with and without clinically active trachoma.

	TF/TI absent (n, %)	TF/TI present (n, %)	Total
ddPCR–ve	957 (98.2)	33 (97.1)	989
ddPCR +ve	18 (1.8)	1 (2.9)	19
Total	975 (100)	34(100)	1009

doi:10.1371/journal.pntd.0004798.t002

PLOS Neglected Tropical Diseases | DOI:10.1371/journal.pntd.0004798 July 12, 2016 7 / 12

IV. TABLE OF CONTENTS

V. INDEX OF FIGURES

VI. INDEX OF TABLES

VII. GLOSSARY OF TERMS

16S	**16 S**vedberg units
AdV	**Ad**eno**v**irus
Bp	**B**ase**p**air
CO	**C**orneal **O**pacity
CoNS	**Co**agulase-**N**egative ***S**taphylococcus*
CoV	**C**oefficient **o**f **v**ariance
C_q	**Q**uantitation **c**ycle
Ct	***C**hlamydia **t**rachomatis*
ddPCR	**D**roplet **d**igital **PCR**
DNA	**D**eoxyribo**n**ucleic **a**cid
EB	**E**lementary **b**ody
EDTA	**E**thylene**d**iamine**t**etraacetic **a**cid
ELISA	**E**nzyme-**L**inked **I**mmuno**s**orbent **A**ssay
EU	**E**valuation **U**nit
FHF	**F**red **H**ollows **F**oundation (Australia)
FPC grading	Trachoma grading scheme based on **F**ollicles, **P**apillae and **C**icatricae
GET2020	**G**lobal Alliance for the **E**limination of **T**rachoma by **2020**
GST	**G**lutathione **S**-**t**ransferase
GTMP	**G**lobal **T**rachoma **M**apping **P**roject
HEp-2	**H**uman **ep**ithelial type **2**
Hi	***H**aemophilus **i**nfluenzae*
Hsp60	**H**eat-**s**hock **p**rotein **60**
IAPB	**I**nternational **A**gency for the **P**revention of **B**lindness
IFN	**I**nter**f**ero**n**
Ig	**I**mmuno**g**lobulin
IL	**I**nter**l**eukin
IPTC	**I**so**p**ropyl β-D-1-**t**hiogala**c**topyranoside
Kb	**K**ilo**b**ase
L	**L**itre
LSHTM	**L**ondon **S**chool of **H**ygiene & **T**ropical **M**edicine
M	**M**olar
m	**M**etre
Mc	***M**oraxella **c**atarrhalis*
MHMS	**M**inistry of **H**ealth and **M**edical **S**ervices
MLST	**M**ulti-**l**ocus **s**equence **t**yping
MMP	**M**atrix **m**etallo**p**roteinase
MOMP	**M**ajor **O**uter **M**embrane **P**rotein
mRNA	**M**essenger **RNA**
NPV	**N**egative **p**redictive **v**alue

NRH	**N**ational **R**eferral **H**ospital, Honiara
NTD	**N**eglected **T**ropical **D**isease
OD	**O**ptical **d**ensity
omcB	**O**uter **m**embrane protein **c**omplex **B**
ORF	**O**pen **R**eading **F**rame
PBPS	**P**opulation-**b**ased **p**revalence **s**urvey
PBS	**P**hosphate-**b**uffered **s**aline
PBST	**PBS**-**t**ween
PCR	**P**olymerase **C**hain **R**eaction
PPV	**P**ositive **p**redictive **v**alue
PZ	**P**lasticity **z**one
qPCR	**Q**uantitative **PCR**
RB	**R**eticulate **b**ody
RNA	**R**ibo**n**ucleic **A**cid
rRNA	**R**ibosomal **RNA**
Sa	***S**taphylococcus **a**ureus*
SAFE	**S**urgery, **A**ntibiotics, **F**acial cleanliness and **E**nvironmental improvement
SNP	**S**ingle **n**ucleotide **p**olymorphism
Sp	***S**treptococcus **p**neumoniae*
TARP	**T**ranslocated **A**ctin-**R**ecruiting **P**hosphoprotein
TE	**T**ris-Cl **E**DTA
TF	**T**rachomatous inflammation – **F**ollicular
TI	**T**rachomatous inflammation – **I**ntense
TMB	**T**etra**m**ethyl **b**orate
TRA	**T**rachoma **R**apid **A**ssessment
TS	**T**rachomatous **S**carring
TT	**T**rachomatous **T**richiasis
UCL	**U**niversity **C**ollege **L**ondon
US CDC	**U**nited **S**tates **C**enters for **D**isease **C**ontrol and Prevention
WASH	**Wa**ter, **S**anitation and **H**ygiene
WGS	**W**hole-**g**enome **s**equencing
WHO	**W**orld **H**ealth **O**rganization
WTSI	**W**ellcome **T**rust **S**anger **I**nstitute

VIII. ACADEMIC ACKNOWLEDGEMENTS

Acknowledgement	Role
Chrissy h. Roberts (LSHTM)	Supervisor
Anthony Solomon (WHO)	Co-supervisor
Martin Holland, Anna Last (LSHTM)	Advisory panel member
Robin Bailey, David Mabey (LSHTM)	Advice and guidance
Matthew Burton (LSHTM)	Advice and guidance. Photograph grading for post-MDA study
Colin Macleod (LSHTM)	Co-leading author on Fiji work; conducted field work and clinical data analysis. Advice and guidance
Michael Marks (LSHTM)	Logistic and administrative support for field work. Advice and guidance
Eleanor Martins, Eleanor Challenger (LSHTM)	Administrative support
Oliver Sokana (Solomon Islands MHMS)	Local Principal Investigator and logistic support in Solomon Islands
Kelvin Jack (Solomon Islands MHMS), Eric Kalae & Leslie Sui (Lata Hospital), Charles Russel (Bellona Rural Health Centre), Suzanne Tetepitu (Honiara NRH)	Solomon Islands field work support
Andrew Velaio (Lata Hospital)	Solomon Islands field work logistic support
Ana Cama (IAPB/FHF)	Pacific-wide field work support, local context
Mike Kama (Fiji MHMS)	Fiji field work support, Fiij study design
Luisa Cikamatana (Laotoka Hospital)	Fiji field work support
Nicholas Thompson (WTSI)	Guidance and study design support of WGS
Alan Walker (WTSI)	Guidance and study design support of 16S rRNA sequencing
Judith Brueur, Rachel Williams, Helena Tutill (UCL)	WGS of *Ct* isolates
Diana Martins (US CDC)	ELISA protocol and reagents
David Nelson (Indiana University)	16S sequencing protocol and reagents
Richard Le Mesurier (FHF)	Study design, historical context for study
Fasihah Taleo (Vanuatu MHMS)	Vanuatu local Principal Investigator
Ratu Bani, Garae Mackline, Annie Bong, Mary Tabi (Vanuatu MHMS)	Vanuatu field work support
Rabebe Tekeraoi (Kiribati MHMS)	Kiribati local Principal Investigator
Raebwebwe Taoaba, Alfred Tonganibeia (Kiribati MHMS)	Kiribati field work support

IX. FINANCIAL ACKNOWLEDGEMENTS

My salary was funded by a Wellcome Trust Intermediate Fellowship to Anthony Solomon (098521). The pre-MDA field work was funded the Global Trachoma Mapping Project (GTMP; ARIES: 203145), funded by the UK Department for International Development via Sightsavers International and the Queen Elizabeth Diamond Jubilee Trust. The pre-MDA laboratory work, post-MDA field work and post-MDA laboratory costs were funded by the Fred Hollows Foundation (FHF; 1041).

All the staff at the FHF have consistently supported the project. My thanks go to Richard Le Mesurier (FHF), Virginia Sarah (International Coalition for Trachoma Control), Joanne O'Sullivan (FHF), Beatrice Varga (FHF), Eva Capa (FHF) and Sarity Dodson (FHF).

X. PERSONAL ACKNOWLEDGEMENTS

My sincere thanks go to Chrissy h. Roberts who has been a constant source of energy and enthusiasm, and has gone above and beyond his role as supervisor to support me throughout the project. I'm indebted to Anthony Solomon for giving me the opportunity to take on this role, and for making time for me throughout the last three very busy years. My thanks also go to Oliver Sokana for unwavering support and companionship in the Solomon Islands and for fueling my appreciation of giant clam and papaya soup. I'm grateful to David Mabey, Martin Holland and Anna Last who have all, through kindness and critique, substantially supported the production of this document.

Tamsyn Derrick, Stephanie Migchelsen, Adriana De Souza Goncalves, Joanna Houghton, Christine Palmer, Sandra Molina, Anastasia Polycarpou, Athumani Ramadhani and Harry Pickering have provided sympathetic ears and forgiving livers throughout the project, and I'm delighted to have worked with them all. Michael Marks and Colin Macleod should also be thanked for their camaraderie and liquor provision both in the UK and abroad. Ana Cama and Richard Le Mesurier have been excellent company throughout various meetings and field trips, I am very grateful for their local knowledge and healthy enthusiasm for eye care in the Pacific.

I'm grateful to my parents Sally and Simon for supporting my academic ambitions both ideologically and financially; I would certainly not have been able to complete the work without them. My future wife Ella has required the patience of a saint to provide such a consistent source of sanity and perspective during this project. This thesis is dedicated to her.

1. INTRODUCTION

1.1 Study context

Visual impairment and blindness constitute a major socioeconomic burden to those affected, the families and carers of those affected, and society at large (1). Trachoma is a chronic keratoconjunctivitis caused by the intracellular bacterium *Chlamydia trachomatis* (*Ct*) and is thought to be responsible for approximately 1.4% of blindness cases worldwide, making it the leading infectious cause of blindness (2). The burden of trachoma was estimated at over 38 million disability-adjusted life years in 2000, with an estimated economic impact of over US$2 billion (3). The magnitude of the trachoma problem galvanised the World Health Organization (WHO) into convening the Alliance for the Global Elimination of Trachoma by 2020 (GET2020) with the specific goal of eliminating trachoma as a public health problem by 2020. This target was subsequently endorsed by World Health Assembly resolution 51.11 in 1998, and has been highlighted as a priority of the VISION 2020 "Right to Sight" initiative (www.iapb.org/vision-2020) (4,5). The persistent will and investment of international partners has yielded significant improvements in our understanding of the epidemiology, pathogenesis and treatment of trachoma over recent decades. However, several challenges remain for the elimination programme. Active (inflammatory) trachoma is thought to be widespread throughout the Pacific Island region, but the prevalence of blinding disease is suspected to be low (6). Local decision makers have questioned whether the aetiology of the observed conjunctivitis differs from trachoma in areas where the blinding sequelae of trachoma are prevalent (R Le Mesurier, personal communication). No insight into aetiology or pathogenesis is offered by the currently available clinical data. This highlights one key issue; namely whether the diagnostic tools available are sufficiently specific to differentiate trachoma from phenotypically similar disease and whether they are appropriate to guide intervention decisions.

1.1.1 Literature search methods

A literature search was conducted with the objective of summarising available data on trachoma and identify key publications that provide the context for the work in this thesis. The primary resource for identifying publications was PubMed, but Embase, Scopus and other databases were utilised when necessary. This literature search used the following search terms individually and in combination: "trachoma", "population-based", "chlamydia trachomatis", ("bacteria*" OR "viral") AND "conjunctivitis", "ocular" AND "trachomatis", "prevalence", "infection", "diagnosis", "Pgp3", "antibodies", "Haemophilus influenzae", "Streptococcus pneumoniae", "immunology", "pacific", "pacific islands" OR "Fiji" OR "Solomon Islands" OR "Kiribati" OR "Vanuatu", "blindness". Reference lists of identified papers were also screened for relevant articles. The literature search was conducted between 2013 and 2016. In some cases (for example, in sections 1.3 and 1.6), multiple references that together support a general concept have been amalgamated and visualised. References where no English language version was available were excluded.

1.2 Natural history of trachoma

Trachoma begins in early childhood and, if not interrupted, can persist over a lifetime (figure 1.2.1). In an untreated community, *Ct* circulates among young children causing conjunctivitis characterised by follicular and papillary inflammation. There can be a period of latency following inoculation where the infection is detectable but no clinical signs are visible. Infection can spontaneously resolve and clinical signs may persist for a period following clearance of infection, as illustrated in figure 1.2.2 (7).

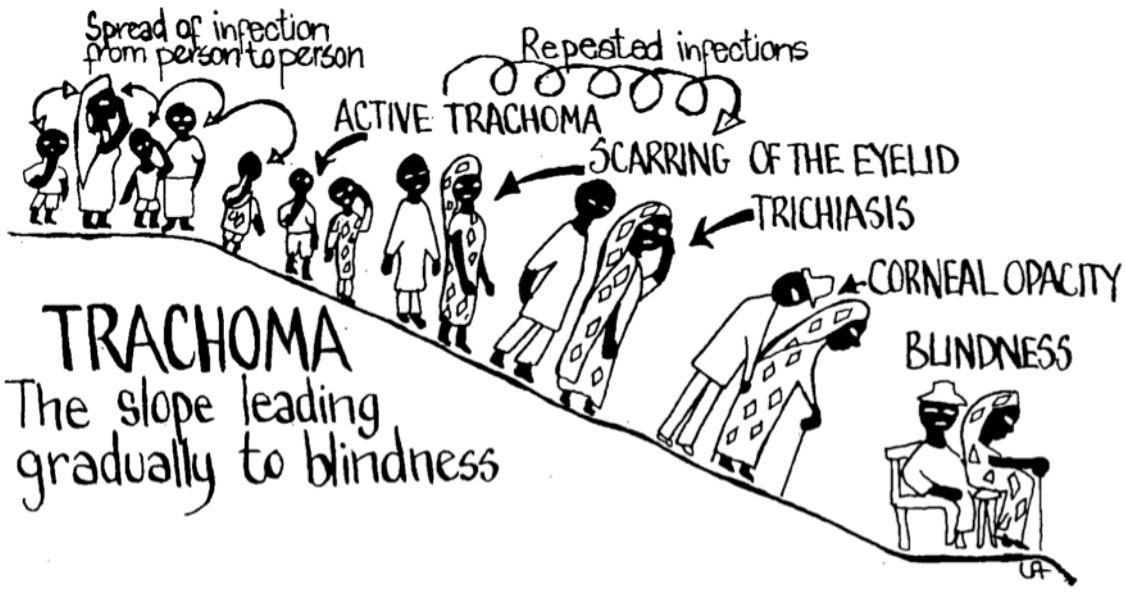


Figure 1.2.1. Cartoon illustrating the natural progression of untreated trachoma. Modified from Francis and Turner (8).

Scar tissue begins to accumulate in white bands on the tarsal conjunctiva after many repeated cycles of re-exposure to and resolution of infection (9,10). Continued inflammatory stimuli (11) cause conjunctival fibrosis to continue throughout a lifetime, eventually becoming so severe that the eyelid is distorted. The distortion of the eyelid can draw the eyelashes out of their normal plane and cause them to abrade the globe of the eye. Severity of disease can range between mild (one or two misdirected lashes) and severe (five or more misdirected lashes) and cases can be progressive (12). Lashes contacting the cornea present the most serious threat to eyesight as the ongoing abrasion can cause damage and subsequent scarring eventually leading to opacity. Corneal opacity (CO) is the blinding stage of trachoma and is effectively untreatable for the majority of people living in at-risk communities.

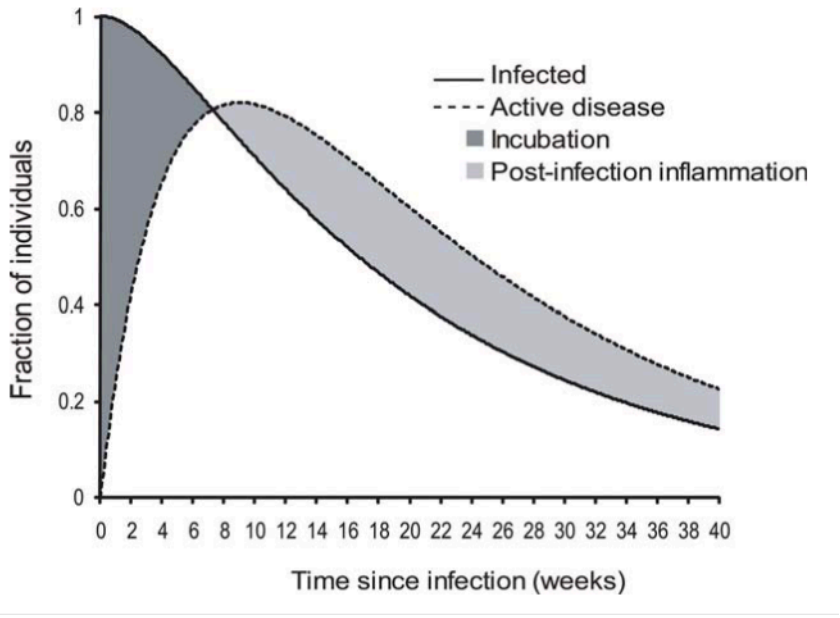


Figure 1.2.2. Modelled relationship between clinical signs of disease and infection. Taken from Grassly and colleagues (7).

1.3 Epidemiology of clinical disease

Trachoma is thought to have been endemic in human populations for many thousands of years; medical records from ancient Egypt, China and Greece describe in-turned eyelashes which are presumed to refer to trachomatous trichiasis (TT) (5,13). In modern times, trachoma has mostly disappeared from developed countries where living conditions, household water facilities and access to health care are improved but remains endemic to many impoverished communities. Over 200 million people are suspected to be living in trachoma-endemic areas, and 1.9 million people irreversibly blind or visually impaired from the condition (14). Estimating the absolute number of people with trachoma is difficult as communities affected are often isolated and poorly served by health care providers. Previous estimates of disease magnitude have been based on a diverse range of studies that used differing methodologies for training trachoma graders and assessing population prevalence. More recently, the Global Trachoma Mapping Project (GTMP) used standardised grader training and survey methodology to reduce the inherent subjectivity of grading and sampling (15); estimates of disease prevalence generated through that project are considered to be more comparable than other estimates.

Transmission of the causative agent of trachoma is thought to be mechanical via flies (particularly eye-seeking species such as *Musca sorbens*) (16,17) and fomites (inanimate mechanical vectors such as cloths or bed linen). Visible secretions around the nose and eyes and flies on the face at time of examination are associated with trachoma (18). Clustering of both disease (19–21) and infection (22,23) are seen at the household and village level. An individual's living environment can influence whether those secretions or vectors to carry them

are prevalent within the community. Uncovered faeces can be a risk factor for trachoma (24), because they provide breeding sites for flies (25). Limited access to water for face washing is another risk factor for trachoma, as it may discourage regular cleaning of ocular and nasal secretions from the face, therefore increasing their chance of facilitating transmission (26).

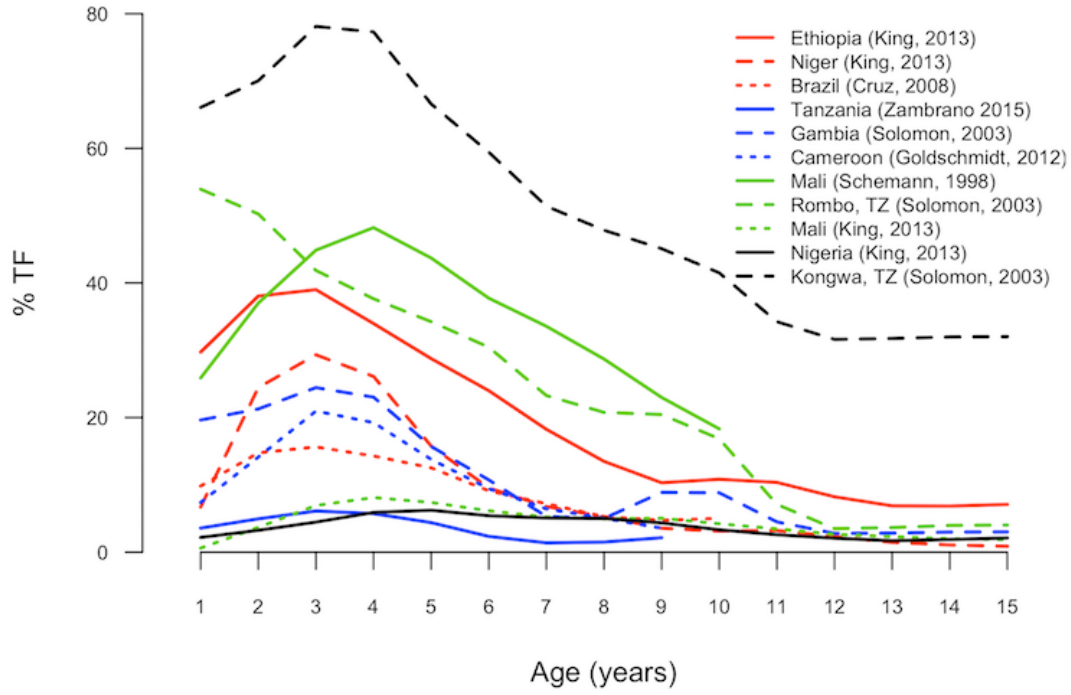


Figure 1.3.1. Age-specific prevalence of trachomatous inflammation–follicular (TF) in children. TF is most common in children aged <5 years old, and peak TF is commonly found in those aged 3-4 years of age. References identified during literature search in December 2015 (27–32).

The highest prevalence of clinical signs of inflammatory disease is found in those under the age of 5 years in many trachoma-endemic areas (30). The age-specific prevalence of TF peaks around the age of 3–4 years (figure 1.3.1). Clinical signs of trachoma were found to have a mean duration of approximately 13 weeks (33), although longitudinal cohort studies have shown this to be highly variable within and between individuals (10). Clinical disease signs, infection intensity and infection duration decrease with age (33). This is presumed to be due to accumulation of partial immunity to *Ct*, although it is not yet clear what constitutes 'immunity' to *Ct*. Trachomatous scarring (TS) typically begins to occur after several years of continuous cycles of re-infection, by late childhood in endemic areas. TS is characterised by visible bands of scarring. In severe cases, this might include Arlt's line, a thick band of scar tissue near the lid margin stretching across the conjunctiva, often seen with other bands of scarring in a weaved pattern in trachoma. The prevalence of scars can reach almost 25% in children aged 1–10 years living in the most severely affected communities (34), although is lower than this in most areas. Cohorts of children in Tanzania have been shown to reach 15-20% scarring incidence

over 5-year study periods (9,10,35,36). Frequency and severity of scarring increases with age (9). Scarring can be highly prevalent in trachoma-endemic communities, for example, in northern Tanzania, where 40.1% of the adult (>18 years) population had evidence of some degree of scarring attributed to trachoma (11), or central Tanzania where 38% of those over the age of 40 years had some evidence of scarring, almost 50% of which worsened over a 5-year study period (9). The most severe conjunctival scars in the population were also found in those over the age of 40 years (9). In an aboriginal Australian community, 45.5% of people over the age of 45 years showed evidence of clearly visible scarring on conjunctival photographs (37).

Most children in endemic communities will experience ocular infection with *Ct* yet not all will develop scarring, and still fewer will progress to TT. Deposition of scarring on the tarsal conjunctiva is driven by a variety of factors over several years, the evidence for which has been recently reviewed (36,38). The reasons for incidence and progression of disease, and especially for differences in susceptibility to these processes, are not clear. Female gender, increasing age, prolonged or regular infection with *Ct* and prolonged, severe trachoma (defined as having trachomatous inflammation – intense [TI] at the majority of study visits) are risk factors for progression from active to scarring trachoma (10,35). Although infection with *Ct* is a risk factor for the incidence of scarring, cases of infection with *Ct* are rarely detected in older people where progression of scarring continues. Progression from mild to severe scarring is associated with severe inflammation. Inflammation in those with progressive scarring was associated with up-regulation of expression of pro-inflammatory genes such as psoriasin (*S100A7*) and chemokine ligand CXCL5 (39). Other nonchlamydial bacteria are associated with inflammation in adults living in trachoma-endemic areas (11).

Trachomatous trichiasis is typically found in older people, and is more frequently observed in women (40,41). TT disproportionately affects those from underprivileged backgrounds (42), and can have a significant impact on quality of life (43). Development of sight-threatening TT is a complex lifetime process and the childhood prevalence of TF is not necessarily reflective of the population burden of TT in a given area. This is evident when examining cross-sectional survey data, where considerable heterogeneity is observed in the relationship between TF in children compared to TT in adults (Spearman's rho: 0.61, $p<0.0001$; figure 1.3.2).

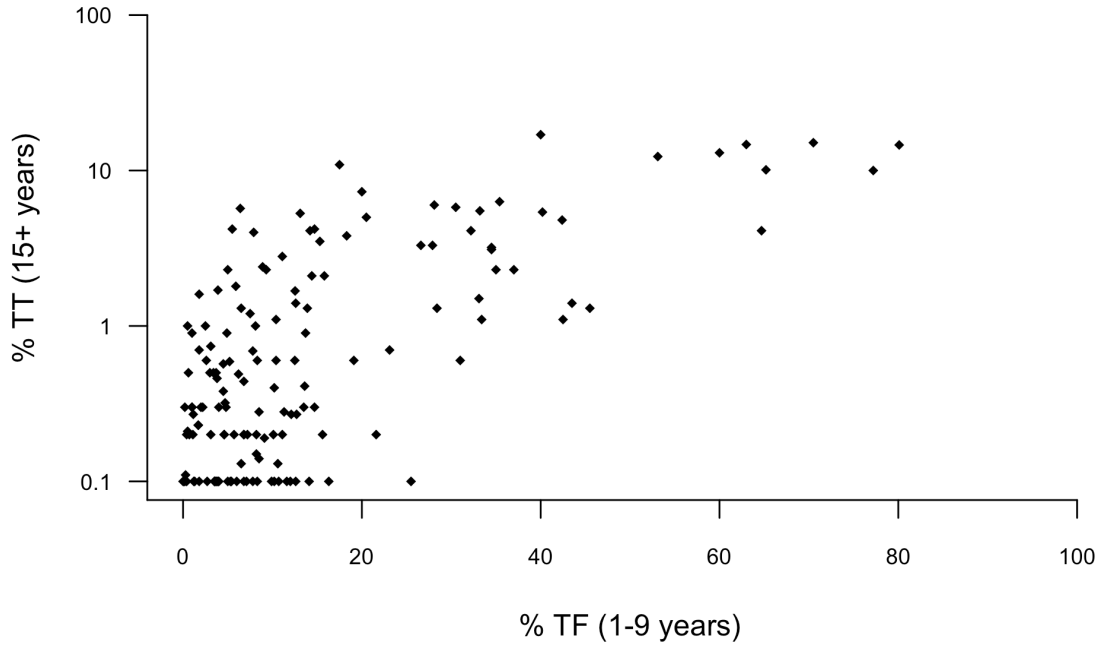


Figure 1.3.2. Prevalence of trachomatous inflammation – follicular (TF) in those aged 1–9 years compared to prevalence of trachomatous trichiasis (TT) in those over aged 15 years. References were identified from PubMed in October 2015 using the terms "population-based" and "trachoma", studies were selected where TT and TF have been assessed in the same population before MDA (24,26,44–65). District-level data were extracted where more than a single district was reported on in a single publication. Not all studies included on the plot presented aged-standardised prevalence estimates, therefore unadjusted estimates were extracted for the purposes of this illustration.

1.4 Classification of trachoma

Trachoma is characterised by clinical assessment of conjunctival disease signs. In the early twentieth century, the ophthalmologist Arthur MacCallan produced the first of several systems to classify trachoma (66). The WHO simplified grading system, a distillation of the complex, multistage process of trachoma into five key signs, has subsequently become the tool of choice for field assessment of trachoma (figure 1.4.1, table 1.4.1). It has been particularly useful as a public health tool because it can be deployed by nonspecialist personnel (67). The simplified trachoma grading system focusses on clinical signs which can be graded reproducibly. While inter-grader agreement for TF and TT are good, TI and TS have been shown to require more training to achieve consensus (67,68). As with many diagnostic systems, cases at the periphery of the diagnostic criteria are often the most difficult to characterise (69). The simplified system lacks detail, and therefore other systems such as Dawson's 1981 system (referred to as the FPC system from here; figure 1.4.1, table 1.4.2), which involves assessment of follicles (F), papillae (P) and cicatricae (C) on the conjunctivae (70) can be necessary for some research applications. When even further detail is required, systems are occasionally modified to suit specific research needs, for example for finer differentiation of severity of follicles and inflammation (71), scarring (9,11) or trichiasis (12).

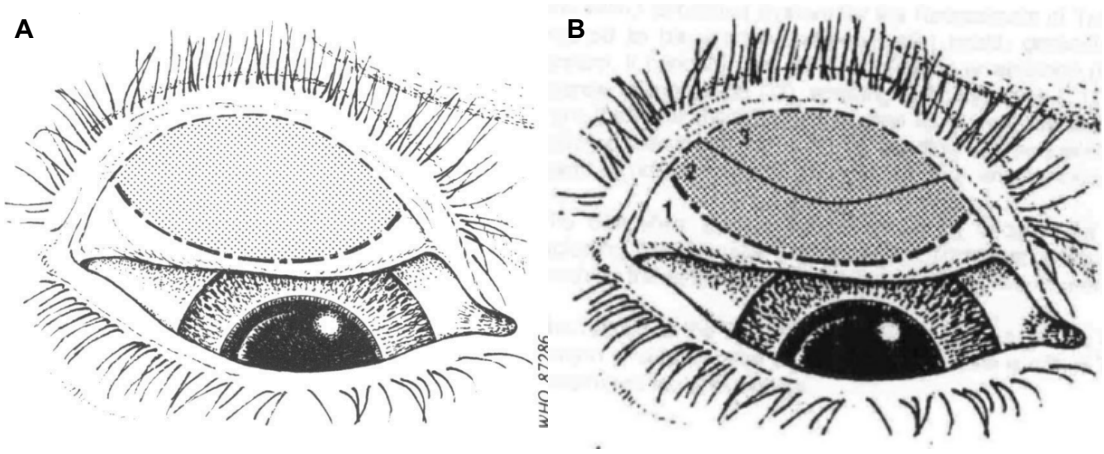


Figure 1.4.1. Zones for grading trachoma using (A) simplified and (B) FPC systems. The clinical signs to be assessed in each of these zones are described in tables 1.4.1 and 1.4.2. Simplified system developed by Thylefors and colleagues (67). FPC system developed by Dawson and colleagues (70). Images created by the WHO and reproduced with permission.

Although the definitions for each trachoma grade are clear, differing interpretation of features of the conjunctiva by examiners make clinical grading subjective. Retrospective re-grading of clinical photographs has been suggested as a quality control mechanism that could reduce the subjectivity of clinical grading. In practice, the agreement between two photograph grades, between photograph grade and clinical grade, and between two clinical grades of the same eye are similar (72,73). Photograding has not been proven to offer additional benefits over clinical grading using the WHO simplified system, and is much less practical. Photography requires sophisticated camera equipment and operator experience, and photograding is time consuming. It is therefore not used at the national programme level. For research purposes or in circumstances where the fidelity of clinical grading may be questioned, a retrospectively gradable record of phenotype is valuable for discussion, and can provide a source for consensus for those grades (particularly those related to severity of inflammation or scarring) that are less reproducible in the field (37).

Not all conjunctival features are included in the simplified grading system. Concretions (benign lipid deposits) and cobblestone papillae (fluid-filled sacs) are two common features that may be mistaken for follicles (figure 1.4.2). Other clinical signs of inflammation have historically been associated with trachoma, such as corneal pannus, where continued inflammation causes blood vessels to encroach the upper corneal limbal margin, or limbal follicles, which give rise to Herbert's Pits after repeated formation. Pannus does not appear to be any more specific for *Ct* infection than follicles (74) (figure 1.4.3).

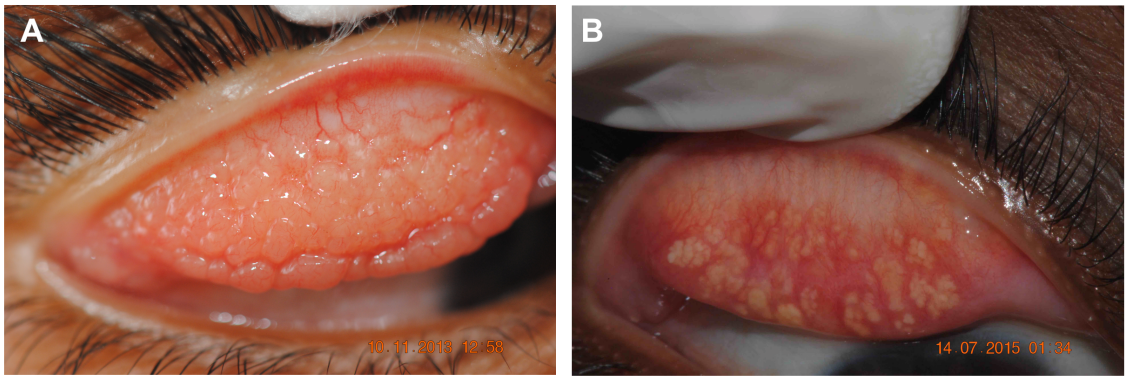


Figure 1.4.2. Trachoma graders should be aware of superficially similar conjunctival features. (**A**) Giant papillae (**B**) Concretions.

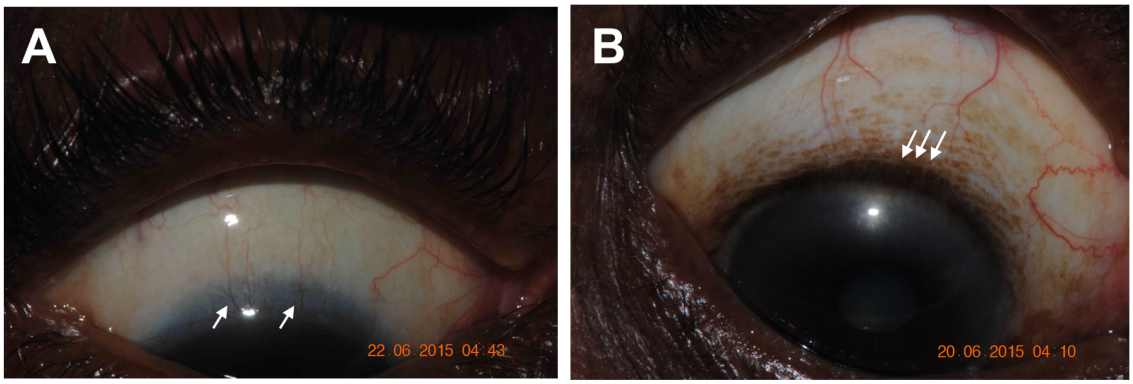


Figure 1.4.3. Clinical signs of trachoma not included in the WHO simplified classification system. (**A**) Pannus (**B**) Herbert's Pits.

Population-based prevalence survey (PBPS) is recommended by the WHO to estimate trachoma prevalence (75). Trachoma is known to cluster at the household and village level, so random sampling at both levels is necessary to achieve the most accurate prevalence estimates. Focussed survey protocols have been developed to determine whether PBPS is warranted. The Trachoma Rapid Assessment (TRA) protocol utilises historical and contemporary records of trachoma to identify suspected high-risk areas for sampling. Schools or community gatherings can be used to recruit individuals for survey to maximise the number of people who can be examined in a short space of time. This protocol should give the assessor the best chance of finding trachoma if it is there, but also does not give all villages, houses or people equal chance of inclusion; these biases preclude accurate estimates of disease burden from this technique (76).

Table 1.4.1. World Health Organization simplified grading system. To meet the criteria for any of these grades, the features must be visible in the central part of the tarsal conjunctiva (figure 1.4.1 A). Reproduced from Thylefors and colleagues (67).

Grade	Definition
Trachomatous inflammation – follicular (TF)*	≥5 follicles of ≥0.5mm in diameter in the central upper tarsal conjunctivae.
Trachomatous inflammation – intense (TI)*	Pronounced inflammatory thickening of the upper tarsal conjunctiva obscuring over 50% of the deep tarsal vessels
Trachomatous Scarring (TS)	Visible scarring in the conjunctiva
Trachomatous Trichiasis (TT)	At least one eyelash rubbing the globe of the eye, or evidence of recent epilation of in-turned lashes
Corneal opacity (CO)	Easily visible corneal opacity over the pupil

*The presence of TF and/or TI is referred to as 'active trachoma'

Table 1.4.2. World Health Organization 'FPC' trachoma grading system. Zones for grading purposes are depicted in figure 1.4.1 B. Reproduced from Dawson and colleagues (70).

Feature	Severity			
	0 (None/absent)	1 (Mild)	2 (Moderate)	3 (Severe)
Follicles (F)	None present	<5 follicles in zones 2 and 3 combined	≥5 follicles in zones 2 and 3 combined but <5 in zone 3	≥5 follicles in each of three zones
Papillae (P)	Normal appearance	Individual vascular tufts prominent, but deep vessels not obscured	More prominent papillae, and normal vessels appear hazy even by naked eye	Conjunctiva thickened and opaque, normal tarsal vessels are hidden >50% of the surface
Cicatricae (C)	None	Fine scattered scars on the upper tarsal conjunctiva, or scars on other parts of the conjunctiva	More severe scarring but without shortening or distortion of the upper tarsus	Scarring with distortion of the upper tarsus
Trichiasis or Entropion (T/E)	None	Lashes deviated towards the eye but not touching globe	Lashes touching the globe but not rubbing in the cornea	Lashes constantly rubbing cornea
Corneal scarring (CS)	Absent	Scarring or opacity but not involving the visual axis, clear central cornea	Scaring or opacity involving the visual axis with the pupillary margin visible through the opacity	Central scarring or opacity with the pupillary margin not visible through the opacity

1.5 Management of trachoma

Globally, the prevalence of trachoma is declining. A proportion of this decline may be secular (due to socioeconomic development leading to improvement of living conditions (77,78)) but can mostly be attributed to successful treatment. Treatment for trachoma involves a package of interventions termed the 'SAFE' strategy, incorporating **S**urgery, **A**ntibiotics, promotion of **F**acial cleanliness and **E**nvironmental improvement (14). SAFE is effective at reducing trachoma prevalence (79–82). Treatment targets *Ct* infection and sight-threatening entropion at the community level. Population-based prevalence of the clinical signs of active trachoma are used as a proxy for the community burden of infection with ocular *Ct* (83), and is the basis of management decisions and elimination targets.

WHO considers trachoma to be a public health problem in districts where ≥0.1% of the entire population, or ≥0.2% of those 15 years-old or above, have TT but have not already been identified and offered management. Patients with TT who refuse surgery, patients who suffer recurrence after surgery and patients awaiting surgery with a date set are excluded from the prevalence estimate with this definition (14). Cases of TT are managed surgically by re-directing misdirected lashes away from the globe. Surgery is effective but does not always offer prolonged benefit, as post-operative recurrent trichiasis is common. Of the two most commonly practised techniques, posterior lamellar is preferred to bilamellar tarsal rotation surgery as recurrence is less common with that procedure (84). Inflammation from bacterial stimuli can associate with recurrent trichiasis in some settings (85,86). Post-surgical treatment with antibiotics has been investigated to help reduce inflammation and recurrence of trichiasis; success of this approach has been mixed (87,88). Alternative nonsurgical options may have similar outcomes for minor cases if used appropriately (89). There is a significant backlog of trichiasis patients (over 3 million cases (14)), the management of which is complicated by barriers to surgery such as lack of trained surgical staff, geographical isolation and fears surrounding the procedure (90).

Districts (administrative regions of 100,000 to 250,000 people (83)) where ≥5% of those aged 1–9 years have TF are also considered to have a public health problem, and intervention is considered to be a priority where the prevalence of TF in the 1–9-year age group is ≥10% (91). At least three rounds of MDA are recommended if the prevalence of TF in children aged 1–9 years is higher than 10%, or five rounds of MDA if prevalence exceeds 30% (91), followed by an impact survey to determine whether further treatment is needed. Macrolide and tetracycline antibiotics are first-line treatments for chlamydial infection. Both are bacteriostatic by directly inhibiting bacterial protein synthesis machinery and have additional positive immunomodulatory effects on host cells. Following oral administration, the tissue concentration of azithromycin is high, so the immunomodulatory effects of this treatment may persist well after clearance from the blood (92). Trachoma can be treated by topical application of 1% tetracycline eye ointment twice daily for 6 weeks. However, treatment adherence is poor and a single, oral dose of azithromycin is equally safe (93), efficacious (94,95) and therefore, in practice, more

appropriate for mass distribution. Mass distribution of azithromycin once annually to at least 80% of a community has been demonstrated to be effective in reducing the prevalence of active trachoma and infection (96–99), and is also thought to reduce incidence of TS, suggesting reduced progression of disease (34,100). The relative expense of the drug is mitigated by a huge (>500 million doses donated, 56 million treatments administered (14)), sustained donation programme of azithromycin (under brand name Zithromax®) from Pfizer, Inc. Azithromycin is a broad-spectrum antibiotic with clinical effects on many common pathogens, including those of *Plasmodium*, *Mycoplasma, Legionella, Neisseria, Haemophilus, Moraxella, Streptococcus, Chlamydia, Treponema* and *Staphylococcus* genera. Perhaps unsurprisingly therefore, MDA seems to have substantial collateral benefits outside of trachoma control, such as treatment of sexually transmitted infections (101), endemic treponematoses (102) and malaria (103). Short-term reduction in episodes of diarrhoeal disease and respiratory tract infections has also been reported (104,105). All-cause mortality has been shown to reduce following MDA programmes (106). No evidence has yet been found that MDA induces antibiotic resistance in *Ct* (107). Conversely, there is some evidence for negative effects of community-wide antibiotic exposure. An increase in macrolide resistance mutations in faecal *Escherichia coli* has been observed up to 6 months post-MDA (108). Carriage of azithromycin-resistant *Streptococcus pneumoniae* (*Sp*) has been shown to be more common in some MDA-treated communities (109,110), but not in others (111). Macrolide-resistant nasopharyngeal *Sp* strains circulating within a treatment-naïve community appear to clonally expand following MDA (112). Where resistant strains were found, they appeared to decline following cessation of treatment (113). It has also been hypothesised that increased detection and treatment of chlamydial infections in the urogenital setting can lead to greater susceptibility to infection by interrupting the natural accumulation of acquired immunity and therefore cause infection incidence to increase (114), although prospective data on this phenomenon is lacking.

Antibiotic treatment is the mainstay of treating *Ct* infection, but following treatment cessation, recrudescence of *Ct* infection and trachoma has been observed (115), thought to be mainly due to incomplete suppression of circulating *Ct* burden or re-introduction by immigrants to the community (116). To maintain suppression of *Ct*, reduction in transmission is targeted by promoting removal of ocular and nasal secretions through facial cleanliness. Promotion of face washing alongside an antibiotic distribution programme compared to antibiotic alone has been associated with a higher proportion of children with clean faces, and clean faces were associated with lower active trachoma (117,118). Trachoma can cause ocular discharge so disentangling correlation and causation is difficult. Environmental improvement is a flexible component of the SAFE strategy, and should specifically target the water, sanitation and hygiene (WASH) needs of a given community. Identifying risk factors such as latrine availability and appropriateness and availability of facilities for face washing can direct environmental improvement programmes to the areas of greatest importance to trachoma. Therefore, the E component of SAFE is highly heterogeneous and location-specific (119). One example of the heterogeneity in interventions and efficacy is latrine provision, which in some circumstances has

been shown to be associated with lower prevalence of trachoma (120) whereas in others it has not (121).

1.6 Ocular infection with *Chlamydia trachomatis*

Intracellular inclusions and follicular conjunctivitis were observed following inoculation of primates (Halberstaedter and von Prowazek, 1907) and later humans (122) with conjunctival scrapings from trachoma patients. *Ct* was cultured in egg yolk sacs directly from conjunctival scrapings from trachoma patients, firstly in Ecuador (although the study was hampered by contamination issues) and subsequently in China (123,124). Originally thought to be a virus, *Ct* is now known to be an obligate intracellular bacterium, and a major ocular and urogenital pathogen of humans. It is closely related to other pathogens of mammals within the *Chlamydiaceae* family. The two-stage life cycle of *Ct* begins when infectious elementary bodies (EBs) bind to the outer membrane of host epithelial cells and enter inside a vesicle, sequestering themselves from the cytoplasm. The vesicles fuse together to form an inclusion, in which the EBs develop into replicative reticulate bodies (RBs) and begin to replicate by binary fission. Within about 48 hours, the RBs return to EBs, and the inclusion is exocytosed into the extracellular space (125).

The epidemiology of ocular *Ct* infection and its relationship to active trachoma has recently been reviewed (126). In most treatment-naïve settings where trachoma is moderately prevalent, ocular infection with *Ct* correlates well with TF at the population level (Spearman's rho 0.86, $p<0.0001$; figure 1.6.1). The association is weaker in low-prevalence (127–129) and post-treatment scenarios (130). Infection is typically more prevalent and present at higher loads in those under 5 years of age than the rest of the population; the majority of active trachoma in a population is also commonly found in that age group. Estimates of the duration of infection in young children suggest it may persist for an average of approximately 4 weeks (33), although, similar to clinical signs, this is highly variable in cohort studies (10). During an infection with ocular *Ct*, ocular and nasal secretions carry infectious bodies (131,132). Ocular *Ct* is thought to be transmitted between people by carriage of these secretions on fingers, flies and fomites. Transmission dynamics of ocular *Ct* can vary seasonally where rainfall is periodic, thought to be due to reduced water availability for face washing in the dry season and reduced fly activity at cooler temperatures in the wet season (133).

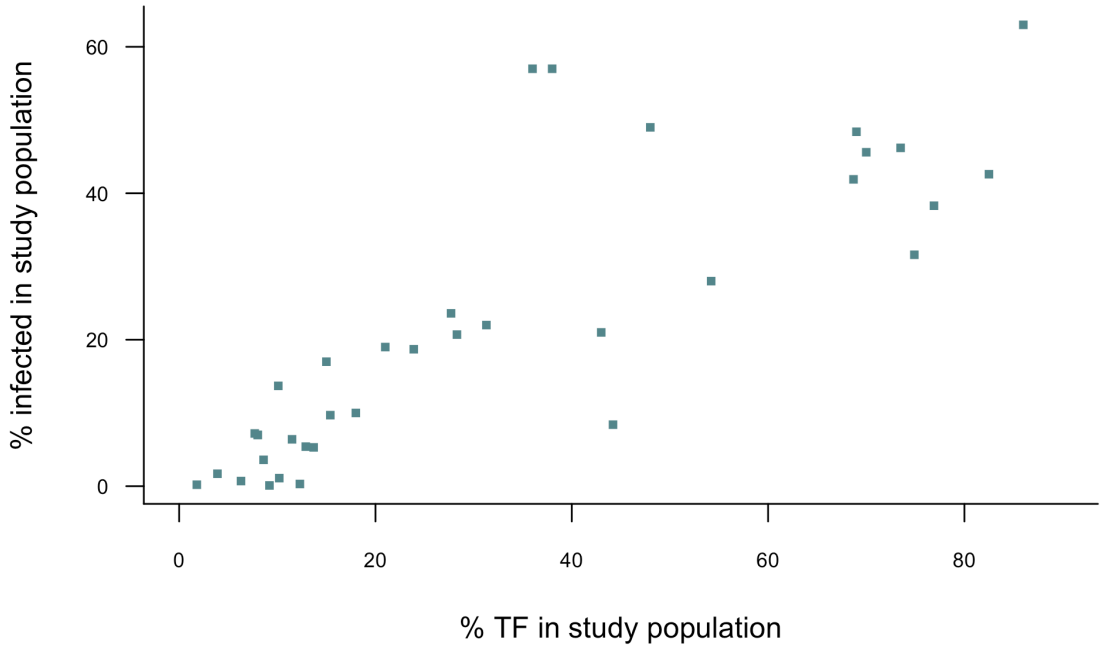


Figure 1.6.1. Population prevalence of ocular *Chlamydia trachomatis* (*Ct*) infection compared to prevalence of trachomatous inflammation – follicular (TF). References identified through literature search using the terms 'trachoma' and 'infection' in December 2015. Studies reporting population prevalence of both TF and *Ct* measured by nucleic acid amplification test in a trachoma treatment-naïve population sample selected for survey for reasons not related to eye health were eligible for inclusion (22,30,31,71,79,97,113,115,129,130,134–151).

Serological profiling of antibodies to the primary chlamydial antigen Major Outer Membrane Protein (MOMP) is used to categorise *Ct* into the ocular serovars A–C, urogenital serovars D–K and lymphogranuloma venereum serovars L1–3 (152,153). Genotyping of the *ompA* gene, which encodes MOMP, yields further variation within the serovars based on evolutionary accumulation of single nucleotide polymorphisms (SNPs), although due to differential rates of evolution to other chlamydial genes, this is not thought to be illustrative of evolutionary relationships (154). Panels of slowly evolving housekeeping genes have been used to further distinguish *Ct* variants (155,156). Multilocus sequence typing (MLST) panels detect numerous genovars within defined serovars, diversity often not reflected by *ompA* sequencing alone (157). The precise mechanism of tissue tropism in chlamydial serovars has yet to be fully elucidated. Ocular strains are unable to make tryptophan, whereas urogenital strains can, synthesising tryptophan to evade interferon (IFN)γ-mediated host tryptophan sequestration (158,159). Recently, *Ct* strains have been isolated from the conjunctiva where the majority of the genome resembles urogenital strains, but *ompA* and some polymorphic membrane proteins (pmps) are more closely related to those from typical ocular strains. The authors suggest this indicates the capacity of *Ct* to recombine with urogenital strains could be a source of re-emergence of ocular infection after clearance (160). However, the children from whom these strains were isolated were very young and were born to mothers recruited into a study of urogenital *Ct* infection, suggesting a distinct possibility that these isolates originated from the mother's genital tract.

Their clinical status, graded using the FPC system, was not consistent with either TF or TI, therefore further work is required to determine the relevance of these findings.

The *Ct* genome consists of a single, circular double-stranded DNA chromosome of just over 1 million base pairs (bp), and a 7.5-kilobase (kb) plasmid (161). The plasmid copy number is variable in cultured strains and in naturally occurring infections. A median 5.3 plasmids per chromosome (range 1-18) was found in West African clinical samples, but no relationship was observed between plasmid copy number and virulence (162). Polymorphism in the *Ct* genome is most common in three major variable regions; the *ompA* gene, the pmps and a plasticity zone (PZ) (163). Recombination is also known to occur throughout the genome (164,165), and whole-genome sequence (WGS) analysis illustrates that it is common (166). To sufficiently enrich DNA from a clinical specimen for successful sequencing, organisms can be cultured. However, multiple passage of organisms through cell lines in the absence of immune pressure could lead to *in vitro* evolution; to mitigate this, culture-independent enrichment techniques have been developed (167,168).

Enhancements in sequencing technology have enabled genetic differences between strains to be defined with very fine resolution. However, it is not known whether pathogen genotype has an influence on trachomatous disease severity. In trachoma-endemic areas, most people are exposed to *Ct*, but not all go on to develop TS and even fewer to TT. Pathogen genetics may play a role (the host factors that may contribute to this are discussed below). Several putative virulence factors have been identified. As a key chlamydial immunogen, MOMP is thought to be involved in virulence (169). It appears to be susceptible to selective pressure, however, no association has been found between genotype and disease severity. The pmps, suspected to be involved in protein trafficking across intra- and extra-cellular membranes, also exhibit increased diversity compared to the rest of the genome and certain subtypes associate with urogenital serovars E and F, which are the most prevalent worldwide, perhaps indicating an advantageous genotype (170). Type III secretion machinery proteins are often associated with virulence in other bacteria; the *incA* component of the *Ct* type III secretion system appears to play a key role in inclusion formation and its absence is associated with attenuated urogenital disease signs (171). Within the PZ, the chlamydial cytotoxin gene is highly variable, and tissue tropism-specific deletions have been characterised (172). The plasmid has been indicated as a major virulence factor in animal models. Plasmid-free urogenital *Ct* inoculation of mice resulted in fewer successful infections than inoculation of plasmid-containing strains (173). Isolates can survive *in natura* with major deletions (174) or complete absence (175) of the plasmid. This indicates the plasmid is not an essential factor for growth and survival, but plasmid-free clinical isolates are rare, suggesting some selective pressure for its retention (176). A plasmid-free *Ct* strain was investigated as a live-attenuated vaccine in macaques, and showed a significant attenuation of clinical disease when compared to inoculation with plasmid-competent strains (177). Cultured ocular *Ct* strains with variations in several genes performed differently in various

in vitro growth measures. Targeted re-sequencing of these genes in Tanzanian clinical isolates replicated the increased variability found, suggesting a role in trachoma pathogenesis (178).

1.7 Non-*Chlamydia trachomatis* microbes and trachoma

A range of bacteria can be isolated from the conjunctiva in trachoma-endemic settings. Bacteria that commonly grow from eye swabs come from genera such as *Corynebacterium, Propionibacterium, Staphylococcus* and *Streptococcus*. Other genera, such as *Micrococcus*, *Bacillus, Moraxella, Pseudomonas,* and *Haemophilus*, are also well represented, although their frequency is more variable (11,129). Culture-independent techniques such as 16S ribosomal RNA gene amplicon sequencing have indicated the microbial community structures at the conjunctiva are similar to those of the skin (179). Bacterial genera such as *Corynebacterium, Propionibacterium, Staphylococcus* and *Streptococcus* also dominate in people not living in trachoma-endemic areas (180). Many less common genera are sporadically detected at the conjunctiva (180–183). Host–microbe interactions at mucosal surfaces are of growing interest. External stimuli, such as contact lens wear, are known to affect the conjunctival flora (184), but significant shifts in microbiome structure in relation to disease have yet to be observed (185,186).

The correlation between TF and *Ct* is reasonably good at the population level prior to interventions against trachoma (acknowledging the methodological limitations described previously), but at the individual level *Ct* cannot be identified from all cases of TF, especially after MDA or where TF is at low prevalence. There are a number of differential diagnoses for follicular conjunctivitis (70). Causes of follicular conjunctivitis can be broadly summarised into: bacterial infection (follicles thought to be more frequent in conjunctivitis caused by species of *Staphylococcus, Haemophilus, Streptococcus* and *Moraxella*), viral infection (adenoviruses, molluscum contagiosum, Herpes Simplex Virus, table 1.7.1), atopic and toxic (cosmetic or topical medications). Some similar conditions are differentiated by additional pathognomic signs, such as giant papillae in atopic conjunctivitis. Others exhibit markedly different demographic distribution, such as folliculitis caused by chemical irritants (make-up or topical medications; affecting people using those products) or ophthalmia neonatorum (vertical transmission of *Ct* and other infections specifically affecting neonates). Zoonotic causes of follicular conjunctivitis include non-*trachomatis* chlamydial infection (from keeping birds, cats and pigs), Newcastle Disease Virus (from poultry) and Parinaud's oculoglandular syndrome (from cat scratches) (70,187,188).

Studies describing non-*Ct* bacteria isolated from the conjunctiva in trachoma endemic settings are discussed in table 1.7.2. *Sp* and *Haemophilus influenzae* (*Hi)* are commonly isolated, however, few studies have been able to show a significant association between non-*Ct* bacteria and clinical signs of disease. In a treatment-naïve, low-prevalence Tanzanian community, *Sp, Hi* type B and *Hi* non-type B correlated more strongly with clinical signs of active trachoma than

Ct (129). In a post-treatment setting, *Sp* and *Hi* type B infection correlated with signs of active trachoma, whereas *Moraxella catarrhalis* (*Mc*; a common cause of ocular inflammation) and *Staphylococcus aureus* (*Sa*) did not (189). Other *Chlamydiaceae*, such as *C. psittaci* and *C. pneumoniae* have also been detected by PCR on the conjunctivae of patients with follicular inflammation in the absence of detectable *Ct*-specific MOMP peptides; these cases were presumed to have arisen from keeping animals (187). Bacteria, such as coagulase-negative *Staphylococcus* (CoNS), *Corynebacterium* (considered commensal during the study), *Sp* and *Hi* type B (pathogenic), were more frequently cultured from adults with TS than those without in a case–control study conducted in Tanzania (11).

It is not clear whether nonchlamydial infections cause prevalent follicular inflammation in their own right, or whether their prevalence in trachoma-endemic environments is related to underlying *Ct* endemicity. In primate models, inoculation of *Hi* (type B or non B) or *Sp* alone did not induce follicles, and did not increase the intensity of follicular inflammation when co-administered with *Ct* (190), so it is possible that inflammation from non-*Ct* infection is influenced by prior *Ct* infection. Several different bacteria are cultivable from conjunctivitis patients in areas where trachoma is not endemic (191). Variation in the relative dominance of *Sp, Hi* and *Mc* at mucosal surfaces can cause inflammation, for example in children with otitis media (192). It is, therefore, theoretically possible for these pathogens to contribute to conjunctival inflammation, but no empirical data from trachoma-endemic settings suggest that disruption of the relative abundance of these pathogens contributes to trachomatous disease prevalence.

Table 1.7.1. Viral causes of follicular conjunctivitis. Distinguishing features not typically seen in trachomatous conjunctivitis are described.

Virus	Virus type	Differentiating features	Ref.
Molluscum contagiosum	dsDNA poxvirus	Conjunctival and periocular mollusca	(193–195)
Adenovirus	dsDNA	Corneal redness, preauricular lymphadenopathy, can be epidemic or associated with acute haemorrhagic conjunctivitis	(196–200)
Herpes simplex virus	dsDNA herpesvirus	Redness, irritation, watering, lid inflammation, follicles more common on lower lid	(201–203)
Newcastle Disease Virus	ssRNA Avulavirus	Zoonotic; associated with turkeys and other poultry	(204)
Measles Virus*	ssRNA Morbillivirus	Conjunctival lesions may resemble follicles. Coinfection with togaviridae can enhance follicular presentation	(205–208)
Mumps virus*	ssRNA rubulavirus	Rare complication of mumps, marked parotid lymphadenopathy	(209,210)
Coxsackievirus	ssRNA enterovirus	Acute haemorrhagic conjunctivitis and associated pathologies	(211)

Ds: double stranded; ss: single stranded.

Table 1.7.2. Studies investigating microorganisms isolated from the eye in trachoma-endemic settings. Study design, method of isolation and association with trachoma have been extracted.

Country (reference)	Design	Size	Specimen collection	Bacteria identification	Outcome	Detected microorganisms	Prevalent microorganisms (>5% in study subjects)	Pathogens associated with trachoma
China (212)	Clinical trachoma cases selected from two Tibetan boarding schools. Sampling frame not specified	35 children aged 6-12 years with trachoma. 60 trachoma negative controls, of whom 9 had less than 5 follicles	Swabbed upper and lower conjunctiva	qPCR for *Ct*, Luminex/qPCR for AdV/HSV/CMV/EBV, culture for non-*Ct* pathogens	25% of TF-negative controls had commensal bacteria, none of them had pathogenic bacteria. 60% of TF cases had *Ct*, of which 57% had a secondary infection with *Sp, Hi, Mc* or *Sa*. Of *Ct*-negative TF-positive children, 1 had AdV, 92% had *Hi, Mc, Sp, Sa* or non-pneumococcal *Streptococci*	*Sp, Hi, Mc, Sa,* non-pneumococcal *Streptococci*, *Arthrobacter*, AdV, *S. epidermidis, Corynebacterium*	Prevalence not clear	No formal analysis of association presented
Gambia (186)	Microbiota of people with various stages of trachoma examined in archived samples from assorted trachoma surveys	Of 130 case-control pairs, 220 samples passed quality control	Tarsal conjunctiva swab collected into RNAlater	*Ct* detected with Amplicor. V3-4 16S amplicon sequencing by 454 pyrosequencing	Microbiome highly varied. Significant variation with age (<10/>10 years) and season (wet/dry). Some differences between people with and without trachoma but not significant	Variable: *Weissella, Rhizobium, Neisseria, Paracoccus, Kocuria, Myceligenerans, Simonsella.* Found in more than 80% of samples: *Bacillus, Staphyloccocus, Propionibacterium, Streptococcus, Corynebacterium, Ralstonia*	*Corynebacterium* (16%)	More *Haemophilus* in people with follicles than those without (not significant). More *Corynebacterium* and *Streptococcus* in those with scarring than those without (not significant)

Gambia (189)	Cross-sectional survey of post-MDA communities to see bacterial correlates of TF after MDA has reduced *Ct* infection prevalence	1538 children aged 0–5 years, 737 who had 3 rounds MDA, 801 who had 1 round MDA. Adjusted prevalence TF is 6.1%	Upper tarsal conjunctiva swabbed for *Ct*, lower fornix swabbed for bacterial culture	Amplicor for *Ct.* STGG medium for transport, streaked onto blood agar (for *Staphyloccus, Streptococcus, Moraxella*) or chocolate agar (for *Haemophilus*)	*Ct, Hi* and *Sp* associated with TF in multivariate model. *Ct* included as independent risk and considered to be confounding	*Sp, Hi, Mc, Sa, Ct*	*Sp* (12.5% adjusted), *Sa* (14.7% adjusted)	*Sp, Ct, Hi*
Nepal (188)	Random selection of people from trachoma-endemic village	101 people aged 1-65 years with TF and/or TI	Upper tarsal conjunctival swab	ArrayTube screening of extracted DNA for multiple chlamydial species	70.2% had at least one species identified. Most single infections were *Ct* alone, but there were 2 single cases each of *C. sius, C. psittaci* and *C. pecorum*. 26 participants had a non-*trachomatis* chlamydial species, either with or without *Ct*	*C. psittaci, Ct, C suis, C pecorum, C. pneumoniae*	*C. psittaci, Ct, C suis, C pecorum, C. pneumoniae*	No trachoma-negative participants screened
Ethiopia (40)	Cross sectional analysis of TT patients >40 years	148 participants of which 101 had TT	Swabbed lower conjunctival fornix	Transported in STGG, cultured in chocolate and blood agar	62% of organism classified as 'commensal'. 79% had at least one organism cultured. TT and female gender was associated with detectable bacteria. *Sp* and *Hi* specifically most closely associated	*Bacillus, Sa,* CoNS, Group A/F *Streptococcus, Abiotrophia,* Nonhaemolytic *Streptococcus*, Gram positive rods, *E. coli, Mc, K. pneumoniae, H. parainfluenzae, Moraxella, Enterobacter*	Viridans group *Streptococcus* (TT: 62.4%, no TT: 34.1%), *Corynebacterium* (TT: 39.6%, no TT 17%), CoNS: (TT: 22.8%, no TT: 8.5%), *Bacillus* (TT: 5.0%, no TT: 2.1%), *Sp* (TT: 15.8%, no TT 4.2%), *Hi* (TT: 18.8%, no TT 0%), Nonenteric Gram negative rods (TT: 18.8%, non TT: 6.4%)	*Sp* and *Hi* associated with TT and female gender. More bacteria recovered from eyes with TT

Tanzania (11)	Case-control study from larger study to investigate nonchlamydial bacterial associations with TS	360 TS cases (>S1b; >18years) and matched controls from larger study	Anaesthetized inferior fornix swabbed for culture	Amies Charcoal medium, streaked onto chocolate and blood agar	Any infection more common in TS cases than controls (54.2% versus 34.4%). More infection cultured from those with more severe scarring	*Bacillus, Hi* type B, *Sp, E. coli, Neisseria, Sa, Aeromonas hydrophilia,* mould fungus, *Actinomyces,* unspecified Gram negative rods, *Enterobacter cloacae, Klebsiella*	CoNS (24.9%), *Corynebacterium* (16.9%) and Viridans group *Streptococci* (9.3%).	CoNS (30.6% vs 19.2%), *Corynebacterium* (24.7% vs 11.7%), *Hi* type B (2.5% vs 0%) and *Sp* (1.9% vs 0.3%) associated with TS
Tanzania (129)	Cross sectional survey of children with trachoma (13.7%) but low prevalence *Ct* (5.3%), aimed to find bacterial and immunological correlates of TF in low-prevalence environment	571 children <10 years	Anaesthetized inferior fornix swab for culture	Amplicor for *Ct*. Swabs transported in Amies charcoal medium and plated onto chocolate and blood agar	*Ct* not associated with TF (6.1% of TFs vs 5.1% of non-TFs). *Sp* and *Hi* type B and non-type B associated with TF more closely	*Bacillus, Micrococcus, Mc, H. parainfluenzae, B. catarrhalis, Neisseria, Enterobacter, Klebsiella.*	Viridans group *Streptotcocci* (32.4%), *S. epidermidis* (14.8%), *Corynebacterium* (9.5%), *Sp* (10.2), *Hi* type B (14%), *Hi* non-type B (9.1%)	*Sp* (21.5% in TFs vs 8.3% in non TFs) and *Hi* type B (24.6 vs 12.6) and non-type B (23.1% vs 6.9%) associated with TF
Gambia (213)	TT patients assessed for bacterial culture and gene expression at 1 and 4 years after surgery to measure recurrence	239 specimens from 426 adults (mean age 58 years) examined at 1 year	First swab from inferior fornix for bacterial culture, second swab from tarsal conjunctiva for *Ct* detection	Amplicor for *Ct.* STGG medium for transport, streaked onto blood agar (for *Staphyloccus*, *Streptococcus*, *Moraxella*) or chocolate agar (for *Haemophilus*).	13.8% had at least one cultured organism, 1% had *Ct*	*Serratia, Klebsiella, P. aeruginosa, Coliform, Moraxella,* Group A *Streptococci*, Viridans group *Streptococci*	Group C/nonspecified *Streptococci* (18%), *Sa* (13%), *Hi* (13%), *Sp* (31%)	No participants without trachoma assessed
Nepal (214)	People living in randomly selected households from one trachoma endemic village	146 individuals of all ages, 28% had TF, 36% had TI, 37% had TS, 20 had TT. 127 swabs were collected	Upper tarsal conjunctiva swabbed	Swabs stored in SPG (DNA extraction and PCR) or M4 transport media (Amplicor)	39% overall Amplicor positive, 31% of TS/TT patients Amplicor positive. Non-*trachomatis* infection accounts for 50% of infection cases in this group	*Ct, C. pneumoniae, C. psittaci*	*Ct, C. pneumoniae, C. psittaci*	*Ct*

Gambia (215)	Two case-control studies: TT cases and matched controls; TS cases and matched controls. Is bacterial infection associated with TT or TS?	121 TT cases and normal controls, 117 TS cases and normal controls	Anaesthetized upper tarsal conjunctiva swabbed for *Ct*, anaesthetized lower fornix swabbed for bacterial culture	Amplicor for *Ct.* STGG medium for transport, streaked onto blood agar, McConkey agar (aerobic), gentamicin blood agar, and bacitracin chocolate agar	37.2% of TT cases compared to 7.4% of TT controls had at least one infection. Infection more common in those with more severe TT. 11.1% of TS cases had infection compared to 6% of TS controls. Many different species detected, *Sp* by far most prevalent. 1/242 TT cases/controls had Ct infection. 44 tested TS case-control pairs negative	*Sp, S. viridans, Streptococcus* group A/C/D/nonspecific, *Sa, P. aeruginosa, Moraxella, Hi, Klebsiella, Neisseria, Coliform, Salmonella, Enterobacter sakazakii*	*Sp* (16.5%), Non-*aureus Staphylococcus* (6.6%)	Infection with any bacteria associated with TT, severity of TT and inflammation
Gambia (88)	Trichiasis patients randomly assigned to trichiasis surgery plus azithromycin or trichiasis surgery alone to see whether azithromycin had an effect on recurrence of trichiasis	204 TT cases receiving surgery plus azithromycin, 222 TT cases receiving surgery	One anaesthetized conjunctival swab for *Ct*, one conjunctival swab for bacteriology	Amplicor for *Ct.* STGG medium for transport, streaked onto blood agar, McConkey agar (aerobic), gentamicin blood agar, and bacitracin chocolate agar	5% of study group had *Ct* infection. Bacterial pathogens were isolated from 29.8% before surgery and 14.9% at 12 months. Infection with *Ct* and any other bacteria not associated with azithromycin administration. *Klebsiella* attributed to the wet season	*Streptococcus* group A/D/nonspecified, *P. aeruginosa, Neisseria*, *Coliform*, Proteus, E. coli, *Salmonella, Serratia, Enterobacter sakazakii,* Fungus	Baseline: *Sp* (42.4%), *S. viridans* (8.3%), *Streptococcus* group C (8.3%), Streptococcus. (8.3%), *Sa* (11.1). 6 months post-surgery: *Klebsiella* (16.3%), *Moraxella* (5.4%), *Sa* (28.3%), *Streptococcus* (5.4%), *S. viridans* (8.7%), *Sp* (20.7%). 12 months after surgery: *Sp* (26.0%), *Streptococcus* (7.8%), *Streptococcus* group C (10.4%), *Sa* (20.8%), *Moraxella* (12%), *Hi* (12%)	Infection was more common as trichiasis severity increased

Nepal (216)	Assessing changes in ocular flora after azithromycin treatment	122 children aged 1-10 assessed before and 2 weeks after treatment with oral azithromycin. 38% had TF	Right upper tarsal conjunctiva swab	Standard culture techniques and antibiotic susceptibility testing	Treatment group has 40% pathogens isolated before treatment, 15% after treatment. Control group has 15% before treatment, 16% after treatment. Reduced prevalence of *Streptococcus*, *Haemophilus* and *Moraxella* 2 weeks after azithromycin	*Moraxella, E. coli, Neisseria*	Pre-treatment: *Sp* (22%) and *Haemophilus* (7.4%); post-treatment (denominator for treated/not treated groups not available): *Sp* (9.2%), *Haemophilus* (5.5%)	Not tested
Nepal (187)	412 conjunctivitis patients screened for *Ct* Hsp60. Seronegative LPS-positive participants chosen as cases, seropositive participants chosen as controls	15 cases, 24 controls	Upper tarsal conjunctival swabs	Gram staining, bacterial culture on blood, chocolate and mannitol agar, viral culture for HSV and AdV, DFA slides prepared for MOMP	No *Ct* DNA in case or control group. *C, psittaci* or *C. pneumoniae* found in 4/15 cases, but not in controls	*C. psittaci, Ct, C. pneumoniae*	Not reported	Not tested
Tunisia (217)	Test use of cytology as tool for diagnosis	151 children with trachoma at first examination, treated and followed up sporadically. 93/927 specimens had inclusions	Kimura spatula from each eye	Spread onto microscope slide and stained	Cytology suitable diagnostic in the absence of facilities for fluorescent antibody staining or culture	Relatively frequent Koch-Weeks bacillus (modern name *Hi* biogroup *aegyptus*)-like slender rods	444/927 smears had at least one visible bacteria, of which 53% were poorly-staining slender rods or coccobacilli (probably *Haemophilus*), 49% densely staining cocci, 35% *Moraxella*-like diplococcus	Not tested
Sudan (218)	Cross sectional microbiological diagnosis of trachoma in the Sudan	46 children with acute trachoma	Blunted pterygium knife scraped twice across the palperberal conjunctiva	First scrape stored in 2-SP and cultured in hens eggs. Second scrape spread onto microscope slide for staining and morphology examination	Over half of trachoma cases	*Sa*	54% had *Ct* cultured in eggs. 21.7% had Diptheroid, 6.5% *S. albus*, 6.5% *E. coli*	No trachoma-negative people examined

Tunisia (219)	Effect of trachoma treatment on purulent conjunctivitis	277 children in two Tunisian villages, 17% had trachoma inclusions on microscopy, >18% had bacterial nontrachomaotus conjunctivitis	Both lower conjunctival sacs swabbed with broth-soaked swab	Culture, Gram stain, antibiotic susceptibility assay, smeared onto slide	Bacterial conjunctivitis reduced by trachoma treatment	Not reported	*Haemophilus* (40%), *Moraxella* (16%), *S. viridans* (65%), CoNS (13%), Diptheroids [*Corynebacterium*] (52%), *Neisseria* (5.5%)	Not tested
Morocco (220)	Samples continuously collected during one year treatment surveillance	Children aged 0-8 selected at random in treatment units	Blunt spatula scrapings of upper tarsal conjunctiva, inner angle of the lids and from the outer canthus.	Scrapings smeared onto slide, Gram stain and morphological examination used for identification	68% of those <2 years had conjunctivitis, 32% of those 2–8 years had conjunctivitis. Subset of 2127 examined for trachoma 72% had Tr-II and 23% had Tr-III	*Neisseria*, *Staphylococci*	*Haemophilus* (81%), *Moraxella* (20.1%), *Sp* (19%), *C. xerosis* (9.0%)	Not tested
USA (221)	Clinical trial of trachoma treatment	140 school children	Fornix swab	Chocolate and blood agar	41% of normal/scarred (Tr-IV) people had at least one bacteria cultured, 43% of people with 'acute' trachoma (Tr-I/II/III)	Not reported	Not reported	Not tested
India (222)	Children recruited over 1 year - method unclear	379 children aged 6 months to 14 years with trachoma. Of those, 102 children had Tr-IV	Lower fornix sampled (method not clear)	Grown on various types of agar	Many different bacterial genera identified	*S. haemolyticus*, Gram negative bacilli, *M. lacunata, Haemophilus*	*Mc, S. albus, Diptheroids, S. viridans, S. pyogenes, Diplococcus pneumoniae*	Not tested

Taiwan (223)	Cross-sectional survey of pre-school and first grade children and their families in three schools to investigate aetiology of trachoma	1027 people of all ages, 135 classified as normal, 156 with nontrachomatous conjunctivitis, 736 with trachoma (Tr-I-IV)	Upper fornix rubbed with loops	Loops directly plated onto blood and chocolate agar. Due to contamination only 5 or more 'pure' colonies for a given species was considered positive	Key genera recovered were group A *Streptococcus*, *Pneumococcus*, *Haemophilus*, *Staphylococcus* which accounts for 30-50% of the isolates recovered. Analysis complicated by seasonal variation in cultured bacteria. Dual isolates counted separately	*Staphylococcus*	Alpha-*streptococci* (11.6%), *Pneumococcus* (16.3%), *Haemophilus* (6.4%)	36.6% of childhood trachoma cases (stage nonspecific) had at least one bacteria cultured, 35.2% of childhood normal controls had at least one cultured. No genera-specific differences

AdV: Adenovirus; CMV: Cytomegalovirus; CoNS: Coagulase-negative *Staphylococci*; *Ct: Chlamydia trachomatis*; DFA: Direct Fluorescence Antibody; EBV: Epstein-Barr Virus; *Hi: Haemophilus influenzae*; HSV: Herpes Simplex Virus; *Mc*: *Moraxella catarrhalis*; MOMP: Major Outer Membrane Protein; qPCR: quantitative PCR; *Sa*: *Staphylococcus aureus*; *Sp*: *Streptococcus pneumoniae*; STGG: skim milk, tryptone, glucose, glycerin; TF: trachomatous inflammation – follicular; TI: trachomatous inflammation – intense; Tr-I/II/II/IV: MacCallan score of trachoma severity.

1.8 Diagnosis of *Chlamydia trachomatis* infection

Basing treatment decisions on clinical signs of disease is helpful for national programmes, as measurement of active disease prevalence requires little specialist equipment or infrastructure. However, MDA is expensive, and resources must be prioritized. Clinical signs of trachoma can be driven by nonchlamydial pathogens and, after several rounds of MDA, can persist in the absence of detectable *Ct* infection at the population level (224). Nucleic-acid amplification tests (NAATs) are much more specific for infection than clinical signs of inflammation, and may provide a cost-effective alternative to clinical signs where unnecessary additional rounds of MDA are avoided (135). There is, therefore, a potential role for molecular diagnostics in making treatment decisions.

Diagnosis of *Ct* infection was originally based on culture and microscopy, which later gave way to the fluorescence-based detection of chlamydial antigens (225) and probe hybridisation of *Ct* DNA (226). Use of NAATs has now been accepted as the gold standard due to their superior sensitivity and specificity (139,227,228). Commercial *Ct* diagnostics are developed for highly sensitive and specific detection of infection at the individual level, and are most commonly used in the urogenital setting. Some commercial tests have been validated for detection of ocular *Ct* infection, for example Aptima Combo 2 (GenProbe) (229) or the Gene Xpert (Cepheid) (230). Both are exquisitely sensitive and offer a diagnostic result that has been globally validated. However, they are closed systems which exclusively use proprietary technology, and are often prohibitively expensive at the programme level. They also offer very limited additional information outside of a binary infection result, meaning the platform cannot be shared between programmes to conserve resources. Using noncommercial open-platform NAATs provides comparable specificity at a fraction of the financial cost. There can be a performance cost to sensitivity, but discrepant results are often the lowest load infections (231), which are thought to be of limited importance in transmission (232). RNA-based tests, such as Aptima Combo 2, are thought to be highly specific and more sensitive than DNA-based tests due to greater abundance of targets (a single organism may have multiple mRNA transcripts per gene copy (233)). Diagnostics targeting the *Ct* plasmid are more sensitive because there are multiple plasmids per *Ct* cell (162). Testing for a fixed copy number target offers comparable specificity and allows, in some formats, the accurate quantitation of genome copies (231).

Long-lived markers of infection, such as antibodies, can also be used to investigate past exposure to a pathogen. Infection with *Ct* elicits a substantial humoral response (234,235), although the functional importance of this response is unclear. Antibodies to *Ct* heat-shock protein 60 (hsp60) have been shown to associate with worse outcomes in pelvic inflammatory disease (236), and with more severe TS (237). Pgp3 has been identified as an immunodominant protein, and a specific marker of prior *Ct* infection in both animal models (238) and human patients (239–241). Enzyme-linked immunosorbent assay (ELISA) for anti-Pgp3 immunoglobulin G (IgG) has shown good comparative performance to anti-MOMP commercial tests in patients with urogenital infections (242). Serological responses to chlamydial proteins

CT694 and Pgp3 from presumed ocular infection has been shown in a trachoma-endemic region of Tanzania (243). At a given cross-sectional time point, a proportion of people living in trachoma-endemic communities will be infected but many more will have been exposed in the preceding months due to ongoing transmission. Therefore, in a *Ct*-endemic community, a high proportion of children are anticipated to be seropositive. For example, in 160 Tanzanian children living in villages to which azithromycin had been distributed, 15% of children had signs of TF, and 49% were positive for Pgp3 antibodies. The proportion of seropositive children can also differentiate between communities of differing trachoma endemicity (244). Longitudinal analysis of seroepidemiology indicates a decrease in anti-Pgp3 and anti-CT694 antibodies 6 months following treatment, indicating some degradation of IgG with time, however, no instances of seroreversion were identified (245). The Pgp3 amino acid sequence is well conserved between *Ct* serovars therefore serological profiling of Pgp3 cannot distinguish prior ocular from urogenital infection; in adult populations seropositivity may sometimes be induced by urogenital infection. Some decrease in Pgp3 seroreactivity was observed up to 6 months after most recent urogenital *Ct* infection, but appeared to stabilise for the longer term (246). By modelling the rate of change in seroprevalence between age groups, rates of seroconversion can be inferred. If a treatment programme successfully reduces infection burden and an F and E programme successfully reduces transmission, the rate of seroconversion should also reduce following implementation of interventions (224). Where these tools have been used in the post-treatment setting alongside NAATs and clinical grading, all three are correspondingly low (247,248).

1.9 Immunology of trachomatous disease

Ocular and urogenital *Ct* infection trigger acute and chronic inflammatory responses, the current understanding of which has been reviewed (249). *In vitro* studies show that initial infection with *Ct* triggers production of pro-inflammatory factors from epithelial cells, such as IL6 and granulocyte macrophage colony stimulating factor (250). Analysis of the conjunctival transcriptome of Gambian children with and without TF highlighted pathways involved in antigen presentation, cytokine and chemokine production and responses, and both NK- and lymphocyte-mediated cytotoxicity. A dominant innate signature was also seen in those with TF (251). Immuno-histochemical analysis of biopsies from Omani children with active disease illustrated mixed inflammatory infiltrate dominated by $CD4^+$ and $CD8^+$ T-lymphocytes at the conjunctiva (252,253). IFNγ is considered the primary mechanism of infection clearance, the source of which is suspected to be Th1 and NK cells (254). Those with TF and *Ct* infection exhibit a slightly altered gene expression pattern with higher levels of inflammatory gene expression. *IL1β*, *TNFα*, *MMP9* and *IL10* are upregulated in TF, whereas infection triggers additional transcription of *IFNγ* and *IL12* p40 subunit (255). Tanzanian children with active trachoma had increased transcription of *S100A7*, *IL17A*, *CXCL5* and *CCL18*, regardless of whether there was a current *Ct* infection (256). Repeated exposure to *Ct* appears to result in accumulation of partial immunity, as frequency (30) and duration (33) of infection reduces with increasing age in trachoma-endemic communities.

A key driver of TS is inflammation. Transcriptome analysis of conjunctival swabs from TS cases and controls showed no association between TS and the T-helper (Th)1 cell-mediated IFNγ-producing phenotype seen in children with active disease. Instead, TS was associated with increased expression of pro-inflammatory mediators such as *S100A7* and interleukin (*IL)1β*, and matrix metalloproteinase (*MMP)7*, *-9* and *-12* (257). In addition, inflammation is a key determinant of post-surgical relapse to TT, with which *S1007A*, among other genes, appears to associate strongly (86). Dysregulation of the *MMP1* to tissue inhibitor of matrix metalloproteinase 1 ratio and increased expression of *S100A7* is also associated with disease recurrence after TT surgery (86,213). Epigenetic factors such as microRNAs have also been shown to be differentially regulated in inflammatory TS cases (258). The processes of inflammation, scarring and trichiasis can progress even after the cessation of episodes of detectable infection (39,85,86,259). Other nonchlamydial infections are associated with trachomatous inflammation, and are thought to drive immunopathology. Some of the suspected pathogens are described in section 1.6 (11,215). The importance of innate defences in the development of scarring was highlighted when the transcription of several *MMP* genes (*MMP7, MMP9, MMP10* and *MMP12*) was found to be up-regulated in patients from whom nonchlamydial pathogens were isolated. The innate immune response may therefore contribute to accumulation of conjunctival scarring in the absence of detectable *Ct* infection (259).

A number of host genetic factors have been identified which influence the outcome of trachomatous disease. Polymorphisms in *IFNγ* (260), *TNFα* (261) and *MMP9* (262) have been shown to be associated with increased risk of severe scarring. Specific Human Leukocyte Antigen (HLA) and Killer Immunoglobulin-like Receptor (KIR) genotype constellations also associated with scarring in trachoma (263). SNPs in genes associated with chlamydial cell entry and host cellular metabolism appear to be differentially present in cases of scarring (264).

1.10 Trachoma in the Pacific Islands

To meet global elimination targets, previously under-represented populations must be sought out and included in worldwide initiatives. Much of our understanding of the biology of trachoma comes from research from a small number of countries, such as Nepal, Tanzania and The Gambia. Very little has been published on trachoma from small Pacific Island countries. Countries in the WHO Western Pacific Region, such as China (212), Cambodia (265) and Australia (266), have incorporated research into their control strategies which shows a clinical picture appropriately diverse given the large economic, cultural and ethnic disparities throughout the region. When the availability and quality of trachoma mapping was summarised in 2005, the Pacific Islands were noted to have particularly sparse data (267). Many of the independent Pacific Island small states, including Solomon Islands, Vanuatu and Fiji, often face significant challenges in provision of basic services and infrastructure to geographically isolated regions, and therefore perform poorly in development indices (268). Indigenous Pacific Islands

populations are dominated by three genetically distinct ethnicities: Melanesian, Polynesian and Micronesian (269). A map of the Pacific Islands is shown in figure 1.10.1.

Trachoma is rarely mentioned in reports of causes of blindness throughout the Pacific. Trauma, cataract and diabetic retinopathy (figure 1.10.2) were among the most common causes of visual impairment in Fiji and Solomon Islands in one literature review from 2002. The same report suggested trachoma to be a major cause of visual impairment in Papua New Guinea, although no evidence was presented to substantiate the claim (270). Pterygium is also common (figure 1.10.2). A small number of cases of trachoma were also identified in Vanuatu and Tonga during surveys of general blindness, although the method for assessing trachoma was not clear (271,272). Trachoma was also uncommon or completely absent in surveys of blindness in the Cook Islands, Samoa, Fiji and Tonga (273–275). Anecdotally, trichiasis patients rarely present to ophthalmic services and surgical outreach programmes (R Le Mesurier, personal communication), although cases have been operated on recently (figure 1.10.3). TT was rarely seen during recent outreach clinics to the Pacific (276).

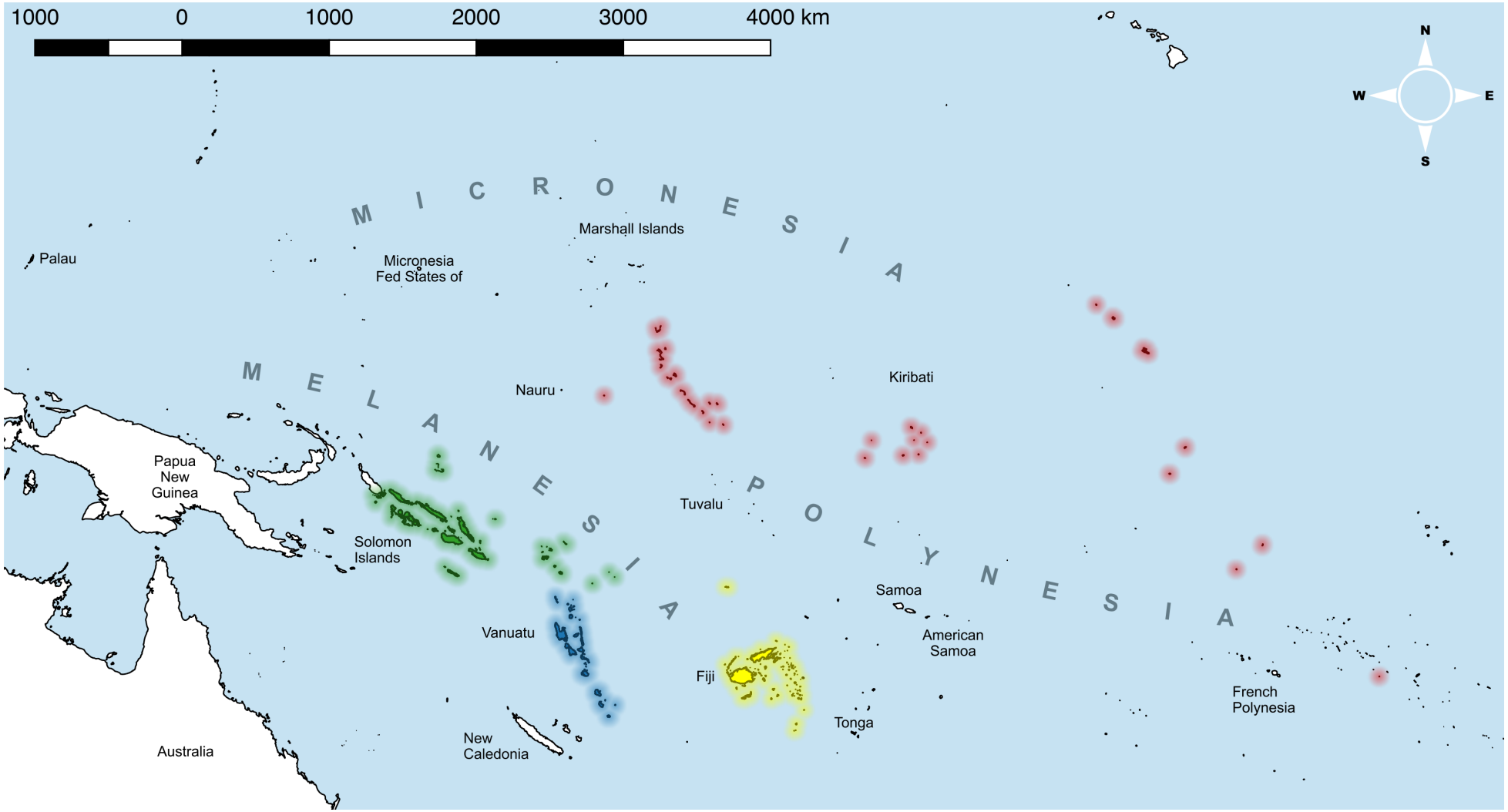


Figure 1.10.1. Map of the Pacific Island small states. Solomon Islands (green), Vanuatu (blue), Fiji (yellow) and Kiribati (red) are highlighted. Prepared using Miller Projection with QGIS 2.16.3 (277). Shapefiles taken from Natural Earth and www.gadm.org.

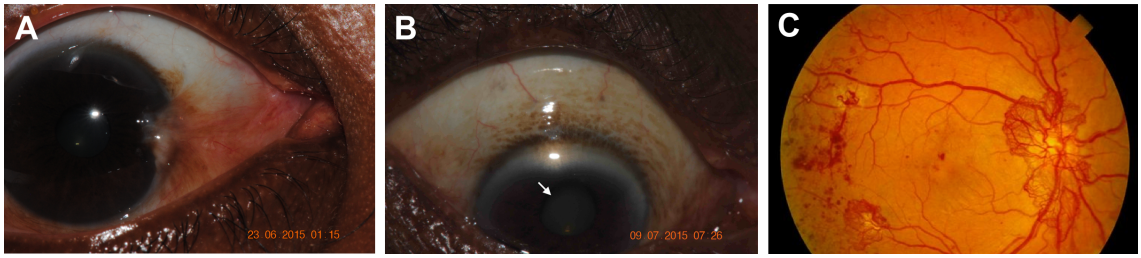


Figure 1.10.2. Common threats to vision in the Pacific Islands. (A) Pterygium, **(B)** cataract and **(C)** diabetic retinopathy.

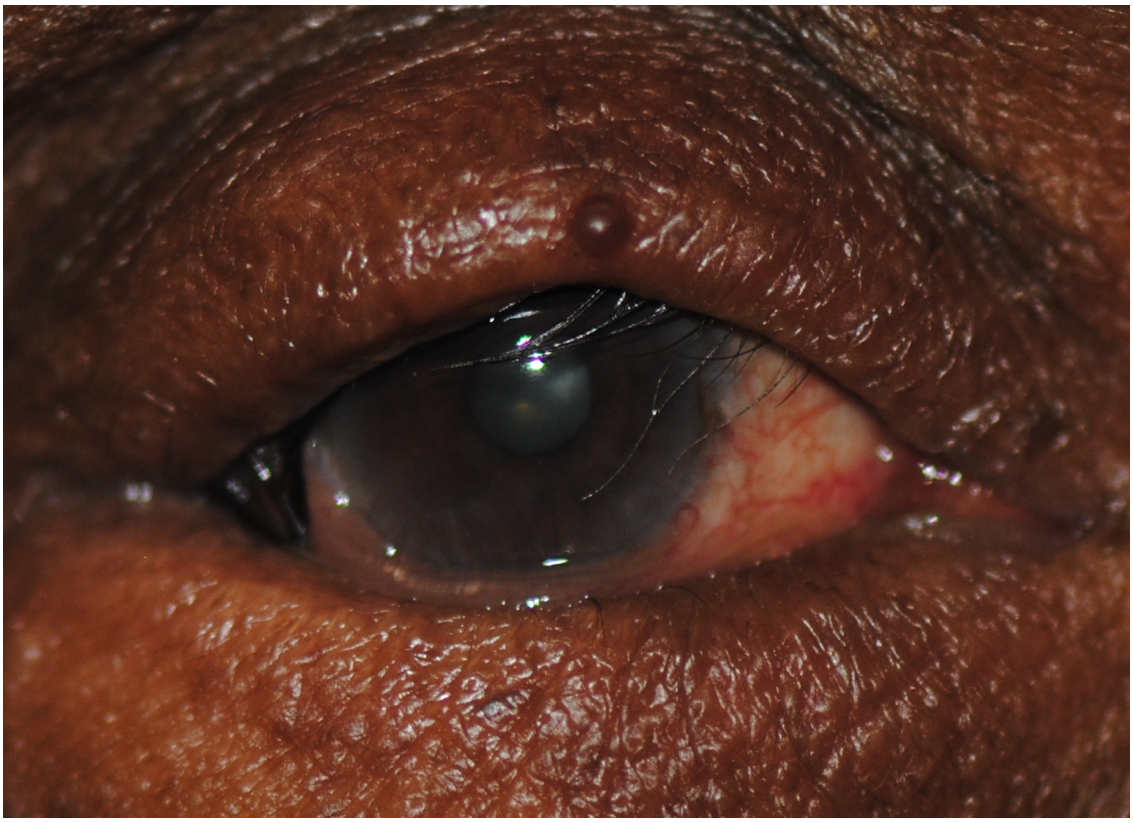


Figure 1.10.3. Right eye of a Solomon Islander from Malaita province who presented for trichiasis surgery in 2016. >10 eyelashes are deviated and contact with the globe of the eye, including over the cornea (Photo credit: O Sokana).

Historical reports have indicated the presence of trachoma in the Pacific region. In 1934, MacCallan amalgamated existing data on trachoma across the British Empire, and noted prevalent conjunctivitis in Fiji, of which he had “little doubt that it is trachoma”. He also noted eight cases of trachoma in the British Solomon Island Protectorate (renamed as the Solomon Islands following their independence from British rule in 1976), and concluded that it “looks as if trachoma were definitely prevalent” (278). Qualitative, observational studies indicated presence of trachoma in Fiji and Papua New Guinea (279–281). Trachoma in Papua New Guinea was noted to be endemic but “of mild intensity and rarely caused visual deficit” (282). Mann recorded a high proportion of examined individuals with trachoma (MacCallan stage Tr I-IV, not separated in discussion) throughout modern-day Papua New Guinea, Fiji, Samoa and Niue during her global expeditions in the 1950s; the method of selection of participants was not clear so no

estimate of prevalence can be made (283). Trachoma has also been mentioned in more recent summaries of neglected tropical disease (NTD) prevalence in the region (284) although accurate prevalence estimates are missing. In response to the requirement for more data, a Trachoma Rapid Assessment (TRA) was undertaken in Nauru, Kiribati, the Solomon Islands, Vanuatu and Fiji in 2007 (6). The findings strongly indicated presence of active trachoma, scarring and trichiasis in the region, and warranted further investigation (6). The average reported rate of trichiasis surgeries at the National Referral Hospital (NRH) in the Solomon Island capital Honiara was four per year over a four-year period (6). Very few studies have investigated ocular *Ct* infection in the Pacific. Bodian observed Giemsa-stained "Prowasek–Halberstaeder inclusions" consistent with *Ct* infection in conjunctival specimens from 42 out of 100 adult Fijians in the 1940s. The clinical status of study subjects was carefully described, with most classified as having active trachoma because of scarring, corneal pannus and inflammation, whereas very few had follicles (285). Ocular *Ct* infections have also been detected in the Pacific region by yolk sac culture in Taiwan in the 1950s (286) and by PCR in Australia (71).

The International Agency for the Prevention of Blindness commissioned PBPSs for trachoma in the Solomon Islands, Fiji and Kiribati between 2011 and 2012 (287). The clinical picture of trachoma in the region was highly variable. Kiribati appeared to have a moderate burden of TT (1.5% in those aged ≥15 years), Fiji had a very high burden of TT (7.4% in those aged ≥15 years), but the Solomon Islands had only three cases, despite over 2000 adults taking part in screening. All three countries were reported to have TF prevalence in those aged 1–9 years between 10% and 25% in study districts. The report also highlighted low levels of improved latrine availability. Interestingly, water availability was found to be good, perhaps due to regular rainfall from the tropical climate. The prevalence of trichiasis in Fiji in particular was surprising given contemporary studies of major causes of blindness specifically reported that trachoma did not appear to be prevalent (275). The GTMP has subsequently supported several surveys in 10 districts across Melanesia (288–290). The data are summarised in table 1.10.1. Of the GTMP survey districts, 6/9 had more than 10% TF in the 1–9 year-olds, whereas 1/9 districts had greater than 0.2% TT in those over 15 years of age (the WHO classification of a public health problem). The GTMP also carried out a survey in Kiritimati, in the east of Kiribati and showed there to be sufficient TF and TT to present a threat to public health, replicating the data from Tarawa in the west of Kiribati.

Table 1.10.1. Current population-based prevalence estimates of trachoma endemicity in the Pacific.

Country (population*)	EU	Examined (1–9 years)	% TF_{1-9}	Examined (15+ years)	% TT_{15+}	Study year (reference)
Papua New Guinea (7,300,000)	South Fly/Daru**	790	11.2	821	0	2016 (288)
	Madang**	1033	9.4	2062	0	
	Southern Highlands (east)**	1391	12.2	773	0	
	National Capital District**	888	6.0	1987	0	
	Southern Highlands (west)**	859	11.7	1579	0	
	West New Britain**	1000	11.4	837	0.5	
Solomon Islands (560,000)	Western**	996	20.4	1679	0.16	2013 (290)
	Choiseul**	881	6.1	1446	0	
	Isabel	1064	24.3	590	0.3	2011 (287)
	Central	772	19.2	1013	0.1	
	Guadalcanal	1000	15.3	-	-	
	Honiara	1026	12.0	-	-	
	Malaita	1066	19.2	-	-	
	Makira	1071	22.3	753	0	
Vanuatu (253,000)	Full country excluding Tafea**	928	12	2511	0.04	2013 (289)
Fiji (881,000)	Northern	1000	20.9	1016	6.2	2012 (291)
	Central and Eastern	1000	10.6	-	-	
	Suva	1000	10.4	-	-	
	Western	1000	19.6	954	8.7	
Kiribati (102,000)	South Tarawa and Betio	999	21.3	959	1.5	2012 (287)
	Kiritimati**	406	28.0	416	0.2	2016 (292)

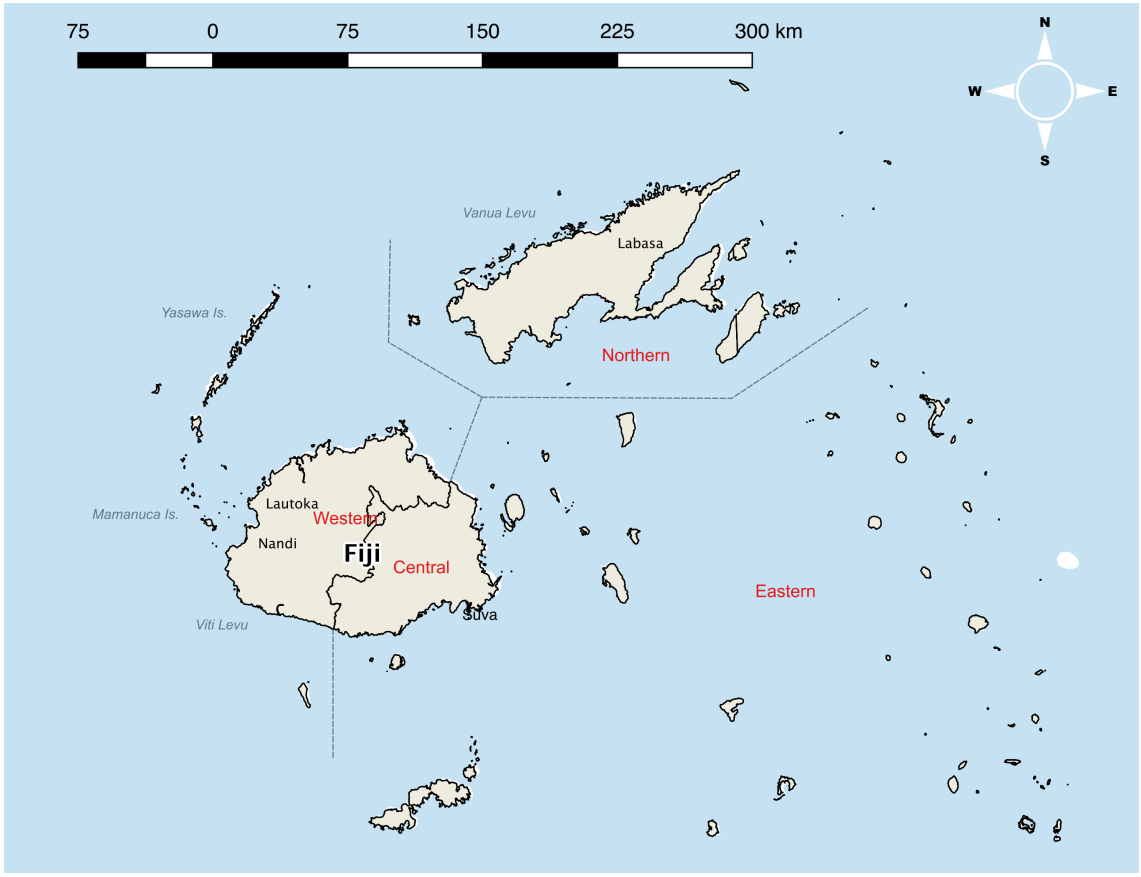
EU: Evaluation unit; TF_{1-9}: Trachomatous inflammation–follicular in those aged 1–9 years; TT_{15+}: Trachomatous trichiasis in those aged 15 years and over.

* 2016 population estimates

** GTMP estimates of prevalence are adjusted for age and gender.

The first part of this thesis includes data on trachoma prevalence in four states of the western Pacific including Kiribati, Vanuatu, the Solomon Islands and Fiji. The remainder will focus on the Solomon Islands and Fiji (figure 1.10.4).

A



B

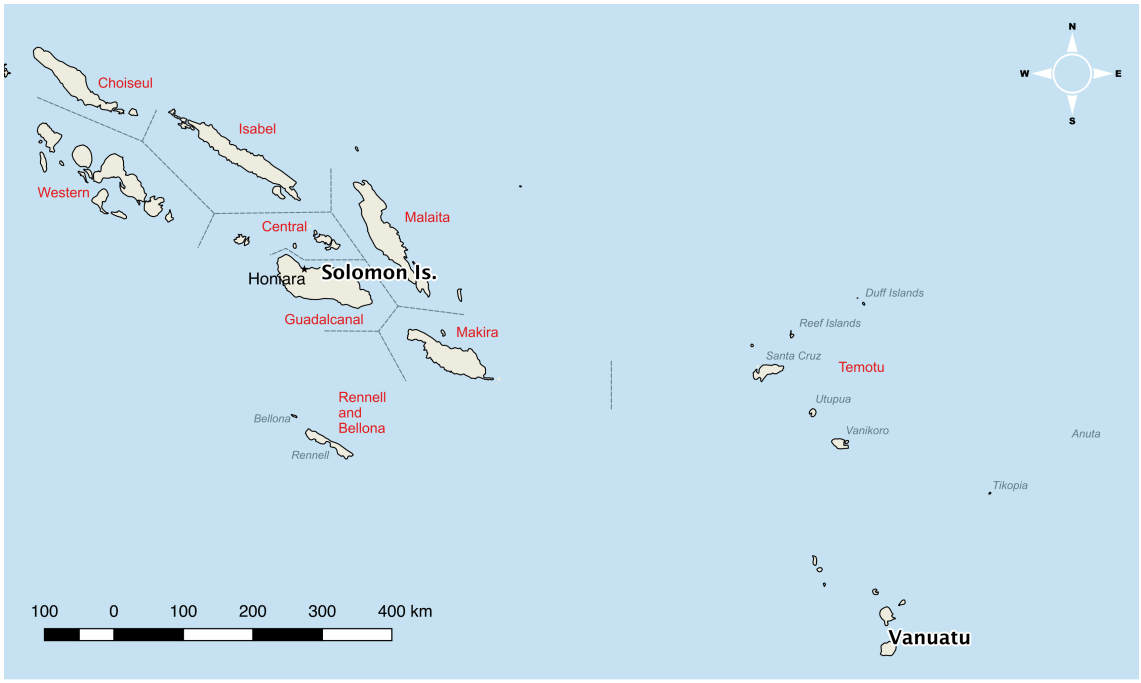


Figure 1.10.4. Provincial maps of (A) Fiji and (B) Solomon Islands. Prepared using Miller Projection with QGIS 2.16.3 [256]. Shapefiles taken from Natural Earth and www.gadm.org.

Both Solomon Islands and Fiji are made up of hundreds of widely dispersed coral atolls and steep-sided volcanic islands. The climate is tropical with warm temperatures and high humidity. In comparison to other trachoma-endemic areas, the Solomon Islands do not experience wide seasonal variation in rainfall (illustrated in figure 1.10.5). Most healthcare services in both countries are provided through the Ministry of Health and Medical Services (MHMS) and healthcare facilities in urban centres, but a substantial proportion of the population live traditional lifestyles with very limited resources in remote, dispersed islands. The Solomon Islands had an estimated population of 515,810 in 2011 (293), and the population is rising due to the high birth rate (estimated at 4.1 births per woman). The population is predominantly of Melanesian (94.5%) or Polynesian (3.0%) ethnicity. Fiji is situated to the south east of the Solomon Islands, has an estimated population of 837,271 in 2012 (294), of which 56.8% are iTaukei (native Fijian) and 37.5% are Indian Fijian. Neither the Solomon Islands nor Fiji had implemented any trachoma-targeted interventions prior to the beginning of the study, however, during the study time period a single round of azithromycin MDA was distributed in the Solomon Islands in the final quarter of 2014, along with a public awareness campaign consisting of newspaper articles (295), radio adverts and public posters (figure 1.10.6). The first round of azithromycin MDA in Fiji is planned for February 2017.

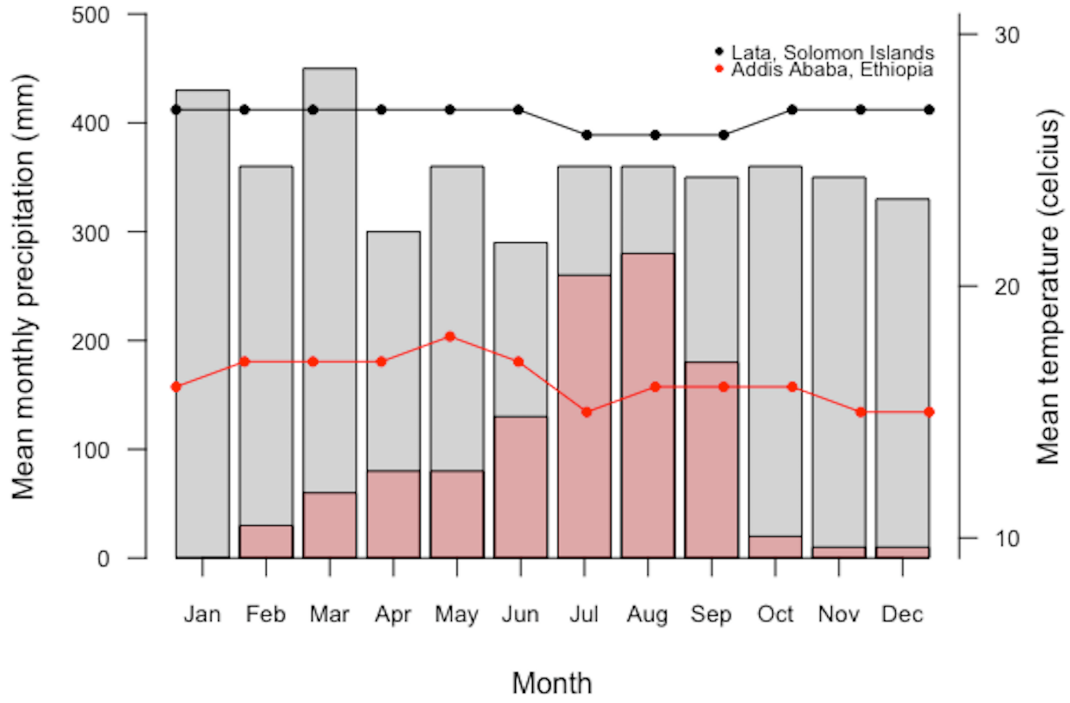


Figure 1.10.5. Temperature and rainfall in Addis Ababa, Ethiopia, and Lata, Solomon Islands. Seasonality in rainfall in is observed in Ethiopia (pink bars) but not in Solomon Islands (grey bars). In both Ethiopia (red line) and Solomon Islands (black line) the mean temperature is consistent all year round. Data are monthly averages from the past 12 years, collected by the Ethiopian National Meteorology Agency and the Solomon Islands Meteorological Services, respectively.

Current data therefore suggest the Pacific Islands present a mosaic pattern of trachoma, with Solomon Islands and Vanuatu having very little trichiasis, Kiribati having moderate trichiasis

warranting intervention, and with Fiji having inconsistent levels of trichiasis between studies. The prevalence of TF is mostly between 10 and 30%, with only one evaluation unit (EU) below the 10% threshold for priority intervention. In Vanuatu and Solomon Islands in particular, the prevalence of TF is sufficient to warrant MDA to most of the population, yet the prevalence of TT is insufficient to be classed as a public health problem. There is uncertainty about the true pattern in areas where data is inconsistent (e.g. Fiji) or unusual (e.g., Solomon Islands) therefore a number of questions must be addressed. These are the source of the hypotheses in chapter two. Firstly, it is important to ascertain whether these findings are reproducible. If so, the burden of ocular *Ct* infection in both Fiji and the Solomon Islands should be ascertained to determine whether light can be shed on the respectively high and low prevalence's of trichiasis.

Figure 1.10.6. Trachoma public awareness campaign poster outside Henderson International Airport, Honiara.

2. STUDY HYPOTHESES AND RATIONALE

2.1 Hypotheses

Figure 2.1.1 is a schematic describing the current conceptualisation of trachoma disease pathogenesis and progression, and highlights the points targeted for assessment in this thesis.

2.1.1 Hypothesis 1

In Melanesia, TF prevalence is above the threshold for intervention whereas TT prevalence is too low to be classified as a public health problem; the previous findings are reproducible and not an artefact of individual surveys.

2.1.2 Hypothesis 2

Clinical signs of TF and ocular infection with *Ct* are not associated in areas where high TF is concurrent with low TT.

2.1.3 Hypothesis 3

One or more alternative pathogens associate more closely with TF than *Ct* does in areas where there is a discordance between TF and TT.

2.2 Rationale

Active trachoma appears to be sufficiently prevalent in Melanesia to warrant intervention with MDA. However, trichiasis appears to be rare, and, in most areas, present at a prevalence lower than WHO's threshold for what constitutes a public health problem. Local policy makers are thereby presented with a dilemma over whether to invest in costly community-wide drug and WASH interventions when trachoma does not appear to constitute a significant threat to vision in this region. Research is urgently required to determine whether there is any evidence of intense transmission of *Ct*, thought to be the principal driver of inflammation that predisposes people to scarring and eventually trichiasis. A better understanding of this problem is critical to local policy, but the decline in the global burden of trachoma will cause the positive predictive value (PPV) of TF as a marker to drop, and therefore this work is also likely to be highly valuable in wider, global-level decisions about which tools are appropriate to characterise disease patterns and guide treatment decisions.

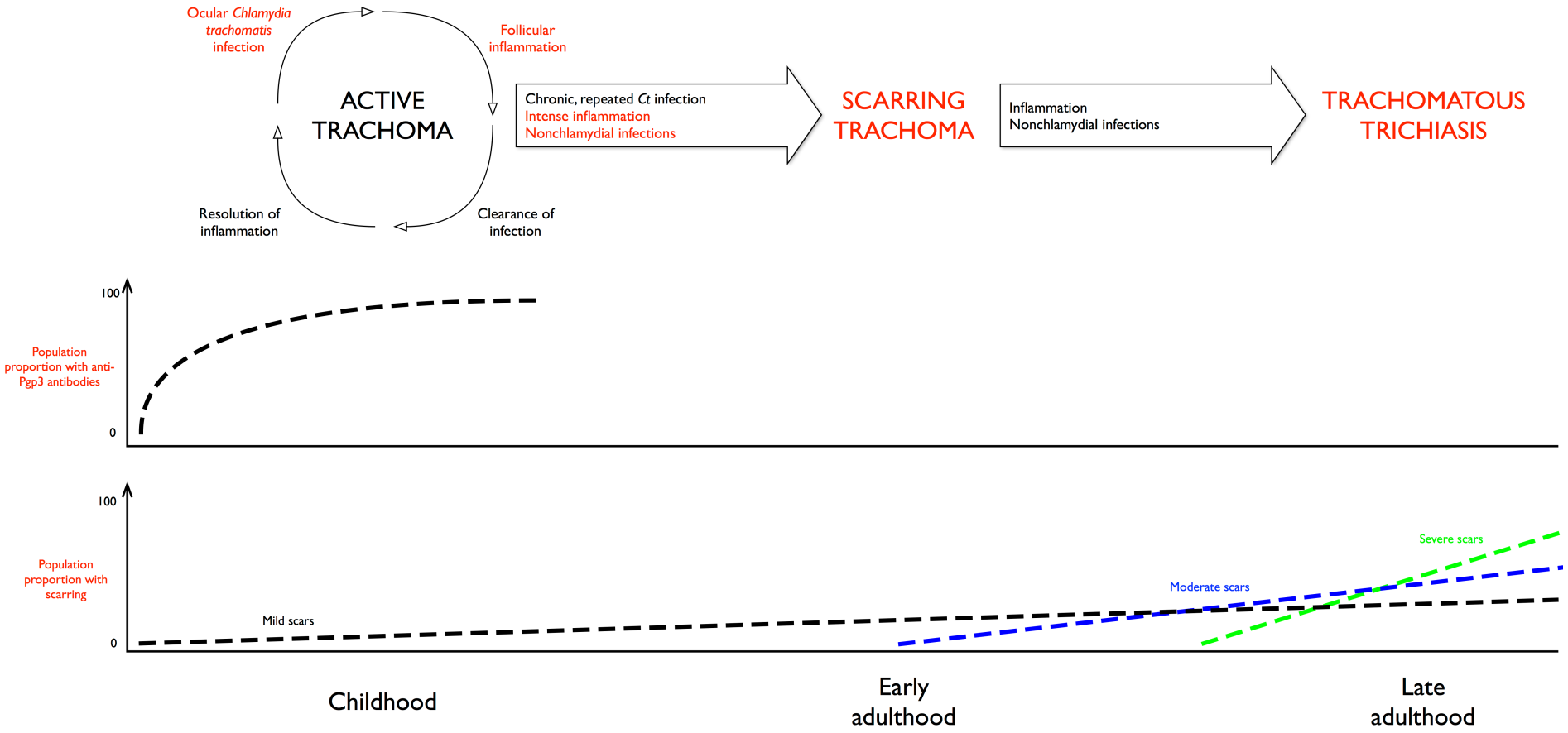


Figure 2.1.1. Key contributors and stages of trachoma pathogenesis. Points of assessment in this thesis are highlighted in red.

3. TRACHOMA IN THE PACIFIC: A CONTEMPORARY OVERVIEW

3.1 Introduction

Reproducible, transparent and consistent survey methodologies are critical to ensuring within- and between-region comparability. There are several Pacific Island countries where trachoma data have, until recently, been very sparse. Historical observational reports are not useful for determining the current burden of disease, and TRA methodology cannot provide accurate population-based prevalence estimates. This chapter sets out to determine whether the clinical picture in the Pacific is reproducibly detected. Clinical data are collected in a series of field surveys. The methodology used is defined, demonstrating that it meets international expectations for generation of high-quality trachoma prevalence data.

3.1.1 Objective

In the Pacific, the trachoma phenotype is suspected to be unusual but given the temporal and spatial variability of disease and the inherent subjectivity of clinical grading, the first objective of this study was to determine whether the phenotype could be reproduced. This data will contribute to the map of trachoma throughout the region. In the Solomon Islands and Fiji, this was done in conjunction with ongoing GTMP activities.

3.2 Methods

3.2.1 Study ethics and consent

The study adhered to the tenets of the Declaration of Helsinki.

Solomon Islands and Fiji surveys dovetailed with GTMP activities, which was covered under LSHTM ethics approval 6319. All surveys, including those where specimens were collected, were additionally approved by the LSHTM Observational Ethics Committee (Solomon Islands and Fiji: 6360, amendment 10145; Vanuatu and Kiribati: 11158). Respective national ethics review boards from the Solomon Islands (HRC13/18), Vanuatu (MOH/DG 01/21 GKT-lr), Fiji (no reference provided) and Kiribati (email dated 25th May 2016) each individually approved the protocol for use in-country.

The study was described verbally to all study participants in local language prior to enrolling. All study participants were required to provide written consent to take part in the study. For those under the age of 18 years, a parent or guardian provided written consent on their behalf.

3.2.2 Study design

Surveys were designed to meet the standards set by the GTMP, or to utilize protocols compatible with the GTMP data collection (15). Two-stage random cluster PBPS were planned.

A number of factors were taken into account when choosing the EU, including provincial and national population, logistic implications of EU geographic distribution, existing programmatic opportunities for collaboration and local knowledge of NTD context. Based on these, the island of Tarawa was selected in Kiribati, rural areas of the whole country of Vanuatu (the larger towns, Luganville and Port Vila, were excluded), the rural areas of Viti Levu (Ra, Ba and Nadroga-Navosa provinces) in Western Division of Fiji and the Temotu and Rennell and Bellona provinces of the Solomon Islands.

International Agency for the Prevention of Blindness surveys in 2012 demonstrated prevalence of TF in children in Kiribati, Fiji and Solomon Islands to be around 20% (287). In an area with endemic *Ct* infection and that prevalence of TF, the prevalence of ocular *Ct* might be expected to be approximately 10% across the region. To ensure homogeneity between studies and with the GTMP, all studies were designed to estimate a prevalence of *Ct* infection of 10% in children aged 1–9 years with a precision of ±3% at the 95% confidence level. The design effect used was 2.65. To account for estimated nonresponse rate, the target sample size was inflated by 10%. Based on those assumptions, 1120 children per EU were needed to meet the sample size. Based on the census data in Solomon Islands, Vanuatu and Fiji, there were 1.2 children aged 1–9 years expected per household and therefore 934 households were needed to meet the sample size (293,294,296). In Kiribati, 1.8 children were expected per household, so at least 623 households were required (297). For the purposes of baseline surveys, one team was considered to be able to examine 30 households per day in Solomon Islands, Vanuatu and Fiji and 25 households per day in Kiribati. A total of 32 clusters were therefore randomly selected in each of the Melanesian EUs and 25 clusters in the Kiribati EU from a list of all clusters in an EU. In Fiji, Vanuatu and Kiribati, probability-proportionate-to-size sampling was used to ensure appropriate chance of inclusion for all clusters based on size. In Solomon Islands, simple random selection was used to select villages as reliable village size data were not available. In each cluster, a list of occupied households was compiled with the help of village leaders and an appropriate number of households were randomly drawn from a hat.

3.2.3 Clinical grading and data collection

In each country, ophthalmic or general nurses were recruited to conduct clinical grading. An internationally standardised grader training scheme, developed as part of the GTMP (15), was used to validate graders. Participating graders attended trachoma theory classes, achieved a kappa agreement score (a measurement of inter-rater agreement between -1 and +1, where -1 is perfect disagreement, 0 is agreement no better than random chance and +1 is perfect agreement (298)) in excess of 0.8 with grades assigned by an experienced trachoma grader when grading photographs, and achieved a kappa score of 0.7 when grading eyelids of school children also graded by a certified grader trainer, who had been validated against a very experienced trachoma grader. Graders were not formally certified for TT grading; kappa scores

were based on TF grade due to the lack of TT cases in school-aged children for validation, however, the definition of TT was thought to be sufficiently clear to allow graders to identify it.

Each participant over the age of 1 year in all selected survey households had both eyes graded for TT in primary gaze and then had both eyelids everted for grading of TF and TI. TS, CO and other ocular morbidities were not systematically assessed in these surveys due to a lack of internationally standardised training schema for them.

Data were collected by dedicated data recording staff using Open Data Kit (ODK) electronic data capture system on Android operating system smartphones. For the pre-MDA Solomon Island and Fiji surveys, the data collection tool was developed externally for the GTMP; for the Vanuatu and Kiribati surveys, a bespoke data collection tool was designed.

In all surveys, conjunctival, photographic and blood specimens were collected as part of other studies, some of which are presented below, and others are part of ongoing operational surveillance programmes.

3.2.4 Data cleaning and analysis

Raw survey data were imported from ODK servers in .csv file format. Data were used anonymously, with a unique study identifier used to link demographic and disease data. Duplicated participant entries and those who either declined examination or could not be examined were removed from the final dataset used to estimate prevalence.

Raw prevalence estimates were adjusted for age using 1-year age bands for the 1–9-year age group, and adjusted for age and gender using 5-year age bands for the ≥15-years age group taken from the most recent available census data.

3.2.5 Role of the candidate

The data presented below result from field studies and analyses that this author led (Vanuatu, Kiribati) or was involved in via collaboration with GTMP activities (Solomon Islands, Fiji). Data generated concurrently that I did not play a role in (for example, Solomon Island GTMP data from Choiseul and Western Province) are summarised in the thesis introduction.

The clinical data collected in the Temotu, Rennell and Bellona in the Solomon Islands and Western Division of Fiji were collected as part of the GTMP. As such, the study design, the planning for the survey, the staff training and the implementation of the survey was primarily a result of collaboration between the respective MHMS in each of the countries and the GTMP Working Group. Data were collected using a version of the bespoke GTMP app that was modified to facilitate collection of specimens. Data for the primary GTMP analysis were curated

and cleaned at the Task Force for Global Health in Atlanta, and owned by each respective MHMS (15).

The infection surveys described in chapters four to seven were embedded within the GTMP studies; I was directly involved in the planning of the infection component of both surveys and therefore indirectly in the execution of the clinical component. I accompanied the team in Temotu, Rennell and Bellona to assist with specimen collection. I performed the age-adjustments presented here independently of the GTMP age-standardisation process with the permission of the Solomon Islands MHMS (the end result was the same). The age-adjusted prevalence estimates from Fiji were generated by GTMP investigators.

The studies in Vanuatu and Kiribati were carried out independently of the GTMP. The projects were conceived by the Pacific Trachoma Initiative meeting in March 2015. The surveys were designed, planned and executed by Chrissy Roberts, David Mabey and the candidate.

3.3 Results

3.3.1 Study demographics

The study enrolled over 12,000 people of all ages prior to MDA from a total resident population of almost half a million people. The sample size required to estimate a TF prevalence with sufficient precision to determine whether MDA is required (10% ± 3%) was achieved in all EUs. In the 1–9-year-old age group, both genders were evenly represented in all four surveys. Men were typically under-represented in the ≥15-years age group. The proportion of the respective national populations covered by these EUs ranged from 5% (Solomon Islands) to 76% (Vanuatu).

Table 3.3.1.1. Descriptive summary of populations surveyed in this study.

			Children (1–9 years)		Adults (≥15 years)	
Evaluation Unit	**Total population***	**Examined**	**n**	**% male**	**n**	**% male**
Solomon Islands (Temotu, Rennell and Bellona)	24,403	3674	1135	53.6	2061	42.5
Vanuatu (excl. Luganville, Port Vila)	176,816	3472	1112	52.5	1941	41.2
Kiribati (Tarawa)	56,284	2922	1036	49.5	1602	31.9
Fiji (rural Viti Levu, Western Division)	184,039	2306	1038	51.7	933	34.5
Total	441,545	12,374	4322	-	6537	-

n: denominator.

* Population estimates from most recent census data (293,294,296,297)

3.3.2 Active trachoma

Trachoma was endemic in study EUs in Solomon Islands, Vanuatu and Kiribati. In each of these EUs, the prevalence was (according to WHO guidelines for trachoma control) high enough to

warrant intervention with at least three rounds of MDA prior to re-assessment. In Kiribati, the prevalence of TF indicated five rounds of MDA should be conducted prior to re-assessment. In Fiji, the adjusted trachoma prevalence was below the WHO target for treatment. In all EUs, very few cases of TI were graded clinically, despite some (such as Kiribati) having substantial burdens of TF.

Table 3.3.2.1. Cases of trachomatous inflammation – follicular (TF) and trachomatous inflammation – intense (TI), and age-adjusted prevalence of TF in children aged 1–9 years.

Evaluation Unit	n	TF (%)	TI ± TF (%)	Age-adjusted TF prevalence	95% confidence interval
Solomon Islands	1135	296 (26.1)	2 (0.2)	22.0	18.5-26.0
Vanuatu	1112	184 (16.5)	0 (0.0)	16.5	14.3-18.7
Kiribati	1036	436 (42.1)	7 (0.7)	38.2	35.7-41.5
Fiji	1038	34 (3.3)	2 (0.2)	2.8	1.4-4.3

n: denominator; TF: Trachomatous inflammation – follicular; TI: Trachomatous inflammation – intense.

3.3.3 Trichiasis

None of the Melanesian EUs studied had trichiasis above the threshold classed as a public health problem by the WHO. In Kiribati, the prevalence of trichiasis was well in excess of the WHO threshold for intervention, which suggests that there is a requirement for surgical intervention as part of the SAFE strategy.

Table 3.3.3.1. Cases of trichiasis and age- and sex-adjusted prevalence of trichiasis in adults aged 15 years and over.

Evaluation Unit	n	TT (%)	Age- and sex-adjusted TT prevalence	95% confidence interval
Solomon Islands	2061	2 (0.1)	0.1	0.0-0.1
Vanuatu	1941	0 (0.0)	0.0	0.0-0.3
Kiribati	1602	18 (1.1)	0.9	0.5-1.2
Fiji	933	0 (0.0)	0.0	0.0-0.2

n: denominator; TT: Trachomatous trichiasis.

3.4 Discussion

The evidence from this study supports the anecdotal and PBPS evidence from the Pacific, suggesting active trachoma is prevalent throughout the region, but prevalence of clinical signs of TT are rare in Melanesia. The clinical scenario in Kiribati seems more severe, with a much higher prevalence of TF, and more cases of TI and TT. Based on this and the previous evidence, SAFE implementation is urgently required in Kiribati. Under current WHO guidelines, Vanuatu, Fiji and Solomon Islands are eligible for intervention with A, F and E components of SAFE. These are costly interventions, and particularly the F and E components require long-term sustained investment in WASH infrastructure. Moreover, entire nations are exposed to broad-spectrum antibiotics, and although evidence does not consistently show a threat of

resistance emergence after MDA, decisions must be well-justified prior to commencement of treatment. In that context, the prevalence of TT in these countries becomes an important factor for consideration. Previous data (6,281) show that cases of trichiasis are found in Melanesia, however, the prevalence seems to be at or below the WHO's definition of a public health problem in all recent PBPS from the region. This raises an important question for how trachoma must be managed here: is trachoma a threat to public health? If not, should programmes invest in costly interventions and community-wide antibiotic exposure to meet guidelines that have not been extensively evaluated in the Pacific Island context? While the simplified system appears to be an excellent tool throughout sub-Saharan Africa to estimate the endemicity status of a district, in Melanesia the evidence generated using clinical grading by this system is inconsistent. Tools are under development for programmatic use to combat the lack of association between clinical signs of trachoma and infection following MDA; these tools may be useful in the Pacific to further characterise disease and define need for intervention. The exception to this pattern is Fiji where the prevalence of TT was previously recorded at almost 8% across the country (291), one of the highest in the world. This estimate is not independently reproducible when using highly standardised techniques, thereby justifying the caution with which this figure was treated by the authors of the original manuscript (291).

The subsequent chapters present data on ocular infections with a view to investigating the potential reasons for the scarcity of TT. The specimens analysed were collected during the surveys in Fiji and Solomon Islands. In chapter four, conjunctival swabs from Fijian children are analyzed to further support the low prevalence of TT by determining whether it is concurrent with prevalent ocular *Ct* infection.

4. OCULAR *CHLAMYDIA TRACHOMATIS* INFECTION IN FIJI

4.1 Manuscript

Previous surveys of trachoma in Fiji had indicated that trichiasis was common, and comparable in prevalence to some of the most heavily endemic districts of sub-Saharan Africa. The following publication considers the burden of *Ct* infection in Fijian children, and indicate that the low estimate of TT prevalence was concurrent with a low prevalence of ocular *Ct* infection (299).

RESEARCH PAPER COVER SHEET

SECTION A – Student Details

Student	*Robert Butcher*
Principal Supervisor	*Chrissy h Roberts*
Thesis Title	*Using alternate indicators to define need for public health intervention for trachoma: Evidence from the Pacific Islands*

If the Research Paper has previously been published, please complete Section B, if not please move to Section C

SECTION B – Paper already published

Where was the work published?	*PLoS Neglected Tropical Diseases*		
When was the work published?	*July 2016*		
If the work was published prior to registration for your research degree, give a brief rationale for its inclusion	*NA*		
Have you retained copyright for the work?*	*Yes*	**Was the work subject to academic peer review?**	*Yes*

If yes, please attach evidence of retention. If no, or if the work is being included in its published format, please attach evidence of permission from the copyright holder (publisher or other author) to include this work.* ***Work published under Creative Commons Attribution 4.0 International Open Access License.*

SECTION C – Prepared for publication, but not yet published

Where is the work intended to be published?	
Please list the paper's authors in the intended authorship order:	
Stage of publication	

SECTION D – Multi-authored work

For multi-authored work, give full details of your role in the research included in the paper and in the preparation of the paper.	*Colin Macleod performed the field work. The study was designed under the GTMP. I worked with Colin and senior author Anthony Solomon to plan the sample collection aspects of the survey. Clinical data were analysed as part of the GTMP. I processed the samples in the laboratory, performed the statistical analysis and generated the tables and figures relating to the infection data. Colin and I wrote the first draft of the paper together, and prepared the first draft of the responses to peer review comments together.*

Student signature: _____ **Date:** 09/12/16

Supervisor signature: _____ **Date:** 19/12/16

PLOS | NEGLECTED TROPICAL DISEASES

RESEARCH ARTICLE

Low Prevalence of Ocular *Chlamydia trachomatis* Infection and Active Trachoma in the Western Division of Fiji

Colin K. Macleod[1,2☯]*, Robert Butcher[1☯], Umesh Mudaliar[3], Kinisimere Natutusau[3], Alexandre L. Pavluck[4], Rebecca Willis[4], Neal Alexander[5], David C. W. Mabey[1], Luisa Cikamatana[3], Mike Kama[6], Eric Rafai[6], Chrissy H. Roberts[1], Anthony W. Solomon[1]

1 Clinical Research Department, London School of Hygiene & Tropical Medicine, London, United Kingdom, **2** Sightsavers, Haywards Heath, United Kingdom, **3** Ophthalmology Department, Lautoka Hospital, Lautoka, Fiji, **4** Taskforce for Global Health, Atlanta, Georgia, United States of America, **5** MRC Tropical Epidemiology Group, Department of Infectious Disease Epidemiology, Faculty of Epidemiology and Population Health, London School of Hygiene & Tropical Medicine, London, United Kingdom, **6** Fiji Centre for Communicable Disease, Ministry of Health, Suva, Fiji

☯ These authors contributed equally to this work.
* colin.macleod@lshtm.ac.uk

OPEN ACCESS

Citation: Macleod CK, Butcher R, Mudaliar U, Natutusau K, Pavluck AL, Willis R, et al. (2016) Low Prevalence of Ocular *Chlamydia trachomatis* Infection and Active Trachoma in the Western Division of Fiji. PLoS Negl Trop Dis 10(7): e0004798. doi:10.1371/journal.pntd.0004798

Editor: Abiola Senok, Alfaisal University, SAUDI ARABIA

Received: November 13, 2015

Accepted: June 2, 2016

Published: July 12, 2016

Data Availability Statement: All relevant data are within the paper and its Supporting Information files.

Funding: Field and laboratory costs of the survey were supported by a Fred Hollows Foundation research award (FHF 1041). Fieldwork was carried out using infrastructure developed as part of the Global Trachoma Mapping Project, whose funding was provided by a grant from the United Kingdom's Department for International Development (DFID) (ARIES: 203145) to Sightsavers. AWS and RB were supported by a Wellcome Trust Intermediate Clinical Fellowship (098521) to AWS. CHR is supported by

Abstract

Background

Trachoma is the leading infectious cause of blindness and is caused by ocular infection with the bacterium *Chlamydia trachomatis* (Ct). While the majority of the global disease burden is found in sub-Saharan Africa, the Western Pacific Region has been identified as trachoma endemic. Population surveys carried out throughout Fiji have shown an abundance of both clinically active trachoma and trachomatous trichiasis in all divisions. This finding is at odds with the clinical experience of local healthcare workers who do not consider trachoma to be highly prevalent. We aimed to determine whether conjunctival infection with Ct could be detected in one administrative division of Fiji.

Methods

A population-based survey of 2306 individuals was conducted using the Global Trachoma Mapping Project methodology. Population prevalence of active trachoma in children and trichiasis in adults was estimated using the World Health Organization simplified grading system. Conjunctival swabs were collected from 1009 children aged 1–9 years. DNA from swabs was tested for the presence of the Ct plasmid and human endogenous control.

Results

The prevalence of active trachoma in 1–9 year olds was 3.4%. The age-adjusted prevalence was 2.8% (95% CI: 1.4–4.3%). The unadjusted prevalence of ocular Ct infection in 1–9 year-olds was 1.9% (19/1009), and the age-adjusted infection prevalence was 2.3% (95% CI: 0.4–2.5%). The median DNA load was 41 Ct plasmid copies per swab (min 20,

the Wellcome Trust Institutional Support Fund (105609/Z/14/Z). NA is supported by the United Kingdom Research Council (MRC) and Department for International Development (DFID) (MR/K012126/1). Funders had no role in the design, performance or analysis of the study, in the decisions on where, how or when to publish in the peer-reviewed press, or in preparation of the manuscript.

Competing Interests: The authors have declared no competing interests.

first quartile 32, mean 6665, third quartile 161, max 86354). There was no association between current infection and follicular trachoma. No cases of trachomatous trichiasis were identified.

Discussion

The Western Division of Fiji has a low prevalence of clinical trachoma. Ocular Ct infections were observed, but they were predominantly low load infections and were not correlated with clinical signs. Our study data suggest that trachoma does not meet the WHO definition of a public health problem in this Division of Fiji, but the inconsistency with previous studies warrants further investigation.

Author Summary

Trachoma, caused by ocular strains of *Chlamydia trachomatis*, represents a major global public health issue, and is the subject of an international elimination campaign. Until recently, data on trachoma in the Pacific Island states have been sparse. The most recent studies have conflicted in their estimates of trachomatous disease burden in Fiji, therefore, surveys using alternative markers (infection testing plus grading) to those already used (grading alone) are warranted to try to shed further light on trachoma epidemiology in this setting. We used an externally validated clinical assessment protocol to show that evidence of active trachoma is present at a low prevalence, and we did not find any cases of trichiasis, the sight-threatening stage of trachoma. From testing of conjunctival swabs with a validated, next-generation PCR, we also found that *C. trachomatis* was present at a low prevalence. Our clinical data suggest that trachoma does not meet the WHO definition of a public health problem in this Division of Fiji, but the inconsistency with previous studies warrants further investigation.

Introduction

Trachoma is the leading infectious cause of blindness, and is caused by ocular infection with the bacterium *Chlamydia trachomatis* (Ct). Trachoma is thought to be a public health problem in 51 countries, with 232 million people at risk of blinding disease [1]. Infection may present as an acute and self-limiting keratoconjunctivitis, but numerous re-infections can lead to potentially blinding sequelae.

Globally, the highest prevalence of active trachoma is found in sub-Saharan Africa [1]. Transmission of infection is thought to be through direct contact with hands or cloths which transfer ocular or nasal discharge between individuals [2], although the bacteria can also be spread by passive contact with eye-seeking *Musca sorbens* flies [3].

Trachoma is diagnosed by clinical examination of the eye. Active trachoma is characterised by the presence of 5 or more >0.5mm lymphoid follicles in the central part of the upper tarsal conjunctiva (trachomatous inflammation–follicular, TF) and/or pronounced inflammatory thickening of the upper tarsal conjunctiva obscuring more than half the normal deep tarsal vessels (trachomatous inflammation-intense, TI) [4]. Scar tissue deposited during resolution of inflammatory disease episodes leads, in some individuals to the misdirection of eyelashes so that they touch the eyeball; this state is known as trachomatous trichiasis (TT) [4,5].

The World Health Organization (WHO) advocates the use of the SAFE strategy–Surgery for trichiasis, Antibiotics, Facial cleanliness, and Environmental improvement, for elimination. Annual mass drug administration (MDA) of the antibiotic azithromycin is recommended for at least 3 years in any district where the prevalence of TF in 1–9 year olds is estimated to be 10% or greater. The decision to undertake MDA is informed by data from a population-based prevalence survey (PBPS) [6] in any district that has been identified as being of concern. The Global Trachoma Mapping Project (GTMP) [7] is currently undertaking PBPSs in all secure probably-endemic districts worldwide, in an effort to complete the baseline trachoma map by the end of 2015.

Cases of trachoma have historically been reported in Fiji [8–11]. A 2007 rapid assessment found a high prevalence of active trachoma in targeted Fijian villages, but no cases of TT [12]. In 2012, a PBPS was undertaken in each of Fiji's four divisions, which estimated division-level prevalences of TF in 1–9 year olds ranging from 10.4–20.9% (19.6% in Western Division). Individuals aged over 15 years were examined only in Western and Northern Divisions, with prevalences of TT in that age group estimated at 8.7% and 6.2%, respectively [13]. The 2012 PBPS results suggested that trachoma was highly endemic in Fiji, and that the prevalence of TT was among the highest in the world. This was in stark contrast to the experience of Fijian ophthalmologists who see cases of TT quite infrequently [12–14].

We conducted a PBPS for TF and TT in the Western Division of Fiji (total rural population 184,039; Fig 1) [15]. In addition we collected conjunctival swabs from children, which were

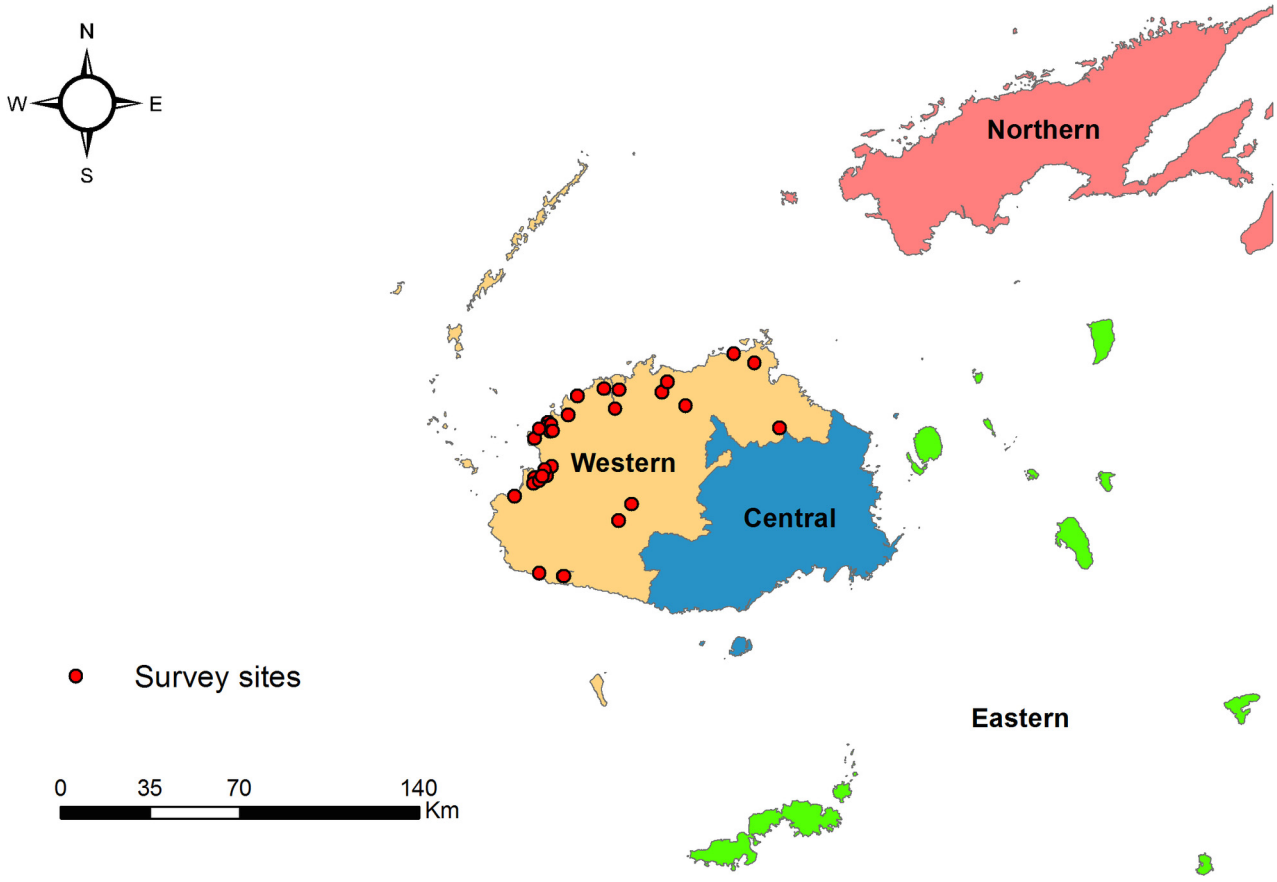


Fig 1. The four administrative divisions of Fiji, with the selected clusters in the Western division marked as points. Prepared using ArcGIS 10.2 (ESRI).

doi:10.1371/journal.pntd.0004798.g001

then processed and subjected to PCR with the aim of estimating the prevalence of ocular Ct infection.

Methods

Ethics statement

The study was conducted in accordance with the Declaration of Helsinki. Consent was obtained from the leader of each community prior to entry into the community. Where possible, village chiefs, local headmen or local leaders were contacted in advance of the survey to gain consent to enter the respective villages. In indigenous Fijian villages, sevu-sevu, a traditional welcome ceremony involving sharing a Kava root-infused water with village leaders, was performed in accordance with the local custom. The study was designed to be paper-free which enabled real-time data upload and review, and streamlined field logistics. In this rural Fijian context, it was considered culturally appropriate for those over the age of 15 to consent for themselves. Verbal informed consent to be examined was obtained from each participant over the age of 15 years. For participants under the age of 15 years, consent for examination and to have specimens collected was given on their behalf by a legally responsible parent or guardian. The Fiji National Research Ethics Review Committee and the London School of Hygiene & Tropical Medicine ethics committee approved this consent protocol. All consent was recorded electronically via the Android phone-based data-capture system [7].

Study design

A cross-sectional, cluster random sample survey methodology was conducted in November and December 2013. Villages identified from local census lists were considered as potential clusters in the sampling. A total 31 villages were selected for inclusion, with 30 households sampled per cluster. The total number of villages was calculated based on the anticipated number of children per household from the latest available census data [15]. In the first stage, after the exclusion of urban centres, villages were sampled with probability proportional to size. At the second stage of sampling, 30 households within a village were selected. Households were selected at random on the day of survey from a list of village households compiled by the village health worker or the village leader. Any person aged one year or more living in a sampled households was invited to participate. Effort was made to ensure participation by absent household members by returning later in the day where possible.

Sample size

The study was powered to estimate a 10% prevalence of ocular Ct infection in 1–9 year olds with absolute precision of ±3% and 95% confidence. A design effect correction of 2.65 was used, based on previous trachoma surveys [7]. We included 10% oversampling in order to account for non-response, the required sample size was 1120 children in this age group. Based on 2007 census data, we expected to find 1.2 children aged 1–9 years per household, therefore we estimated 30 households from each of 31 clusters would be sufficient to recruit 1120 children. The overall sampling methodology was in accordance with that used in the GTMP [7].

Data collection

Data were collected on an Android smartphone using a slightly modified version of the GTMP LINKS app, which is an implementation of the Open Data Kit toolbox for mobile data collection efforts (https://opendatakit.org/) and has been described elsewhere [7]. GPS coordinates were recorded for each participating household. The age and sex of each household member

was then recorded, along with a record of consent to examination, refusal or absence at the time of the survey.

Clinical assessment

A single GTMP-certified [7] trachoma grader examined both eyes of each participant using a 2.5× binocular loupe and sunlight. Each eye was assessed for the presence or absence of TT, TF and TI, according to the WHO simplified grading system [4,7]. An individual trained in the use of the Android phone application recorded results. Disposable gloves were used during swab collection, and alcohol hand gel was used between individuals to prevent carry-over contamination from one subject to the next. Participants found to have active trachoma were provided with a course of 1% tetracycline ointment and directions in its method of application. Participants found to have any significant ocular pathology were referred to the nearest eye care centre for management.

Conjunctival sampling

For each participating child aged 1–9 years, a specimen was taken from the right upper tarsal conjunctiva with a polyester swab (Puritan Medical Products, ME, USA) and using a standardised collection procedure [16]. The specimen was taken immediately after clinical grading and the swab was immediately returned to its packet, secured and labelled with an anonymised five-digit numeric code. Swabs were kept in the field in a cool, dry container and were then air-dried overnight, before being transferred to 5°C storage the following morning; they were then maintained at this temperature until processing, between 1 and 5 months later.

Control swabs

Fifteen negative field control swabs were collected by passing a swab within 15 cm of the eyes of a seated subject, chosen by random selection from the list of all specimen labels prior to commencement of the survey; specimen labels were used sequentially. Positive control swabs were prepared by briefly submerging swab heads in an homogenized solution of Ct strain A2497[17] elementary bodies and cultured hep2C cells at a dilution factor of 1 in 500, suspended in a phosphate-buffered saline. 15 such positive control swabs were prepared in London and stored in 2 mL Eppendorf tubes, then frozen and retained at LSHTM, UK. 15 further positive controls were prepared in the field, and were stored at 5°C until analysis. Field control swabs and swabs from study subjects were indistinguishable, and laboratory staff were masked to swab status.

DNA extraction

Genomic DNA was extracted in to 50 μL nuclease free water using the Norgen Genomic DNA Purification kit (Norgen Biotek, Canada) according to manufacturer's protocol. For quality control, a sample with DNA extracted from a clean swab was included in each extraction batch.

C. trachomatis infection testing

A Ct-specific droplet digital PCR (ddPCR) assay was used according to a published protocol [18], and with the minor modification that an 8 μL aliquot of DNA was used in each reaction. Briefly, each well contained 1X ddPCR supermix, 0.2 μM fluorescent probes and 0.9 μM forward and reverse primers for *Homo sapiens RPP30* and Ct plasmid ORF 2. Thermal cycling conditions were 95°C for 10 minutes, followed by 40 cycles of 95°C for 15 seconds and 60°C for

Table 1. Primer and probe sequences for *C.trachomatis* targets and control using ddPCR. [19]

Molecular target and primer or probe	Nucleotide sequence and modifications
Homo sapiens RNase P/MRP 30-kDa	
subunit (RPP30) (endogenous control)	
RPP30-F	5′ AGA TTT GGA CCT GCG AGC G 3′
RPP30-R	5′ GAG CGG CTG TCT CCA CAA GT 3′
RPP30_HEX_BHQ1	5′ HEX-TTC TGA CCT GAA GGC TCT GCG CG-BHQ1 3′
C. trachomatis cryptic plasmid	
pLGV440 (circular; genomic DNA; 7,500 bp)	
Ct-plasmid-F	5′ CAG CTT GTA GTC CTG CTT GAG AGA 3’
Ct-plasmid-R	5′ CAA GAG TAC ATC GGT CAA CGA AGA 3’
Ct-plasmid_FAM_BHQ1[b]	5′ 6FAM-CCC CAC CAT TTT TCC GGA GCG A-BHQ1 3′
Ct-plasmid_HEX_BHQ1[c]	5′ HEX-CCC CAC CAT TTT TCC GGA GCG A-BHQ1 3′
C. trachomatis (serovar A) *omcB* gene	
Ct-*omcB*-F	5′ GAC ACC AAA GCG AAA GAC AAC AC 3′
Ct-*omcB*-R	5′ ACT CAT GAA CCG GAG CAA CCT 3′
Ct-*omcB*-FAM-BHQ1	5′ 6FAM-CCA CAG CAA AGA GAC TCC CGT AGA CCG-BHQ1 3′

a MRP, mitochondrial RNA processing endoribonuclease; 6FAM, 6-carboxyfluorescein reporter; BHQ1, black hole quencher 1; HEX, hexachlorofluorescein reporter.

b C. trachomatis plasmid probe used in screening (first) assay.

c C. trachomatis probe used in quantitative (second) assay.

doi:10.1371/journal.pntd.0004798.t001

1 minute; then a final hold for 10 minutes at 98°C. A modified *omcB* probe was used to improve quenching efficiency and limit background fluorescence (Table 1).

Specimens from persons with TF and/or TI were tested for the presence of Ct *omcB*, a well-conserved genomic target, to ensure that cases of infection were not missed due to insertion/deletion or recombination events disrupting the site of the diagnostic primers for plasmid DNA [20].The number of plasmids per chromosome was also assessed using the method described by Last et al [19]. A single well was run for each sample.

Statistical analysis

Data analysis was carried out using R [21]. Observed cluster-level frequencies of TF were adjusted for age in one-year age-bands using data from the 2007 Fiji census [7,15]. Confidence intervals were calculated by bootstrapping adjusted cluster-level proportions [22]. A binomial confidence interval was used for the upper bound of the TT prevalence estimate [23]. ddPCR data were analysed using QuantaSoft software (BioRad, Hemel Hempstead, UK). A positive ddPCR result was defined as one having a greater than 95% confidence in a non-zero load under a Poisson approximation, as described elsewhere [18].

Results

Descriptive epidemiology

A total of 413 households were visited over 31 clusters. No data were collected on non-participation of households. We enumerated 2306 individuals for inclusion in the study, of whom

1038 were aged 1–9 years, 335 were aged 10–14 years and 933 were aged 15 years and over. Ten (0.4%) individuals declined consent to participate; 2296 were examined. The median age of those examined was 11 years (mean: 20; min: 1; 1st quartile: 5; 3rd quartile: 32; max: 91), and 1289 (56.1%) were female. Following data cleaning, 1009 children were included in the study, which is very close to the targeted sample size of 1018.

Clinical assessment

Data records without paired clinical and swab data (n = 29/1038) were discarded from the analysis. TF was observed in 34/1009 (3.4%) 1–9 year-olds. The age-adjusted prevalence of TF was 2.8% (95% CI 1.4–4.3%;). TI was observed in 2/1009 (0.2%) 1–9 year-olds. The age-adjusted prevalence of TI was 0.1% (95% CI: 0.0–0.3). No cases of TT were observed in 928 examined participants aged 15 and over. The age-adjusted prevalence of TT in those aged 15 years and above was 0% (95% CI 0–0.2%).

In addition to the 34 cases of TF found in those aged 1–9 years, 6.4% (21/330) of those aged 10–14 years and 1.1% (10/928) of those aged 15 years and over were found to have clinical signs of TF and/or TI. The median age of those with TF was 8 years (mean: 11; min: 1; first quartile: 5; third quartile: 11; max: 83).

Ocular *C. trachomatis* infection

A total of 1038 children aged 1–9 years had ocular swabs taken and analysed. 16 (1.5%) swabs were unusable due to labelling errors. Of the 1022 remaining, 13 (1.3%) failed quality control because there was no detectable endogenous human target. 1009 (97.1%) specimens passed quality control (>95% confidence in non-zero human *RPP30* load). The mean droplet number per well was 14062 (first quartile 12634, median 13837, third quartile: 15247). The median endogenous control load was 9576 *RPP30* copies/swab. 19/1009 (1.9%) tested positive for the presence of Ct plasmid DNA. 1/34 (3.1%) children with active disease tested positive for Ct DNA and 18/977 (1.8%) children without active disease tested positive (Table 2). There was no association between cases of TF and cases of infection (p = 0.644 Mantel-Haenszel Chi-square). No new cases of infection were detected when active disease cases were retested with a multiplex plasmid/*omcB* ddPCR assay.

The median load of infection in positive specimens was 41 Ct plasmid copies/swab (min 20, first quartile 32, mean 6665, third quartile 161, max 86354). Among the 19 swabs that tested positive for the plasmid, seven additionally tested positive for presence of *omcB* in the multiplexed plasmid/*omcB* test. The mean plasmid:chromosome ratio was 4.4 plasmids per chromosome (range 1–12), consistent with the findings of Last et al [19]. The age-adjusted prevalence of ocular Ct infection in 1–9 year-olds was 2.3% (95% CI: 0.4–2.5%).

Control swabs

Note A - page 75

All 30 positive control swabs (15 field and 15 lab) tested positive for Ct. The field control swabs had a 58.7% reduction in mean Ct plasmid load as compared to those stored frozen. Mean Ct

Table 2. Qualitative test for infection in persons with and without clinically active trachoma.

	TF/TI absent	TF/TI present	Total
	(n, %)	(n, %)	
ddPCR–ve	957 (98.2)	33 (97.1)	989
ddPCR +ve	18 (1.8)	1 (2.9)	19
Total	975 (100)	34(100)	1009

doi:10.1371/journal.pntd.0004798.t002

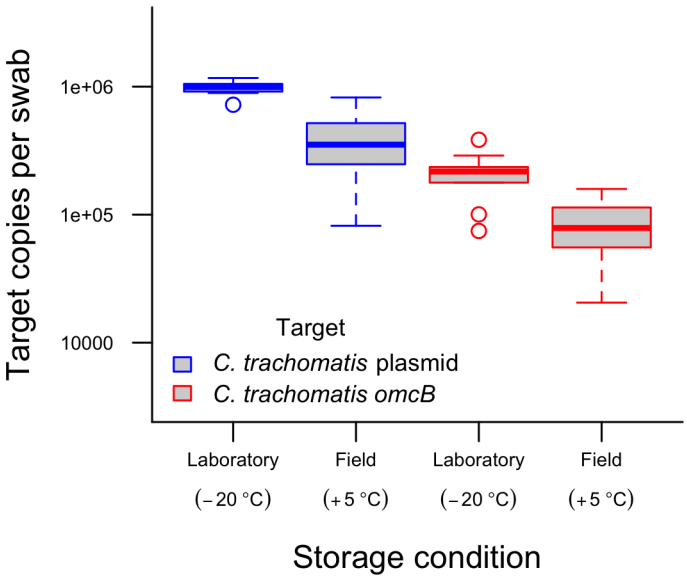


Fig 2. Comparative Ct *omcB* and plasmid load recovered from swabs stored at -20°C (n = 15) and +5°C (n = 15) for the duration of the study (November 2013 –July 2014).

doi:10.1371/journal.pntd.0004798.g002

omcB load for the field storage group was also reduced by 58.7%. The reductions in mean load of both plasmid and *omc*B were statistically significant (Pearson's Chi-squared test p = 0.0002 and 0.000016 for difference in plasmid and *omcB*, respectively)). The mean load of human DNA from hep2C cells was 45% lower in the frozen swabs than in the swabs that were stored in the field, but this difference was not statistically significant (80 vs 148 copies/swab; p = 0.18) (Fig 2). Of the 15 negative control swabs collected in the field, 1 swab was lost in transit. The other 14 negative control swabs tested negative for both human and Ct DNA.

Discussion

We estimate there to be a low prevalence of active trachoma and a correspondingly low prevalence of ocular Ct infection in the Western Division of Fiji. These data represent the first data on ocular Ct infection in the Pacific Island small states and represent a significant step towards improving knowledge of trachoma in the region.

We found no association between infection and active trachoma. This result is unsurprising, given the generally low correlation between signs of disease and ocular Ct infection in low prevalence settings [24–27]. It has been suggested that this may be because where Ct prevalence is low, other pathogens may be associated with the active trachoma phenotype [27,28]. It is possible that nucleic acid amplification testing may miss some low-load Ct positive samples due to the relatively high sampling variation when an analyte is at very low concentrations. Some commentators have suggested the sensitivity of this ddPCR assay may be too low for trachoma programs [29] due to the sensitivity observed in a 'face value' diagnostic evaluation by Roberts and colleagues. However, it was demonstrated that the discrepant results occurred in a mathematically predictable manner related to the analyte concentration and that most PCR-based technology will share an absolute limit to the number of analyte copies per test that will be reproducibly detected. It was highlighted that in a traditional discrepant analysis the sensitivity of this ddPCR assay could have been as high as 98% [30] and we therefore believe the test was appropriate in this setting.

For logistical reasons, swabs that were collected during this survey were not frozen during storage. A number of studies have illustrated that host [31] and chlamydial DNA [32,33] are stable in the short term when stored dry. Evidence from our positive control swabs indicates that a substantial proportion of chlamydial and human DNA is lost during storage over a few months at 5°C. However, this will only result in loss of qualitative diagnostic accuracy at very low loads of infection, consistent with the findings of Dize and colleagues [34]. In our positive control swabs, the ratio of plasmid to chromosome targets was the same regardless of storage conditions, indicating a similar rate of degradation between both genome components. We described above the difficulties caused by sampling error when diagnosing very low load infections, and degradation during storage may have caused previously detectable samples to become not reproducibly detectable. Improving specimen transport and storage conditions may have resulted in a closer association between clinical signs of disease and Ct infection. However, as low-load infections may be poorly associated with TF [16] and of limited importance in driving transmission at community level [35], we consider their detection not to be critical. The loss of sensitivity is however a limitation when considering the findings of this cross-sectional prevalence study.

We did not find any cases of TT in this population. This is in contrast to the previous (2012) PBPS, which found a high prevalence of TF (19.6% in 1–9 year-olds) and TT (8.7% in $\geq$15 year-olds) in this Division [13,36]. A 2009 rapid assessment found communities in which a high proportion of examined children had TF, but–like the present work—no cases of TT [12]. The source of these discrepancies is the subject of on-going research but we have observed social practices of eyelash epilation in Fiji that may have been misdiagnosed as trachomatous trichiasis.

The number of children per household was higher in this survey than in the 2007 national census, and we therefore reached our sample size in a lower number of households than expected. Data were not collected on households not enrolled in the study, nor on household demographics that may have explained why our sample size was reached with fewer houses than originally thought. However, the target sample size was very nearly achieved, and our individual participation rate was over 99%, therefore the risk of attrition bias is considered to be low. The difference in TF prevalence between the present data and the 2012 PBPS could be due to poor consensus between graders, seasonal variation in trachoma prevalence or an artefact of the cross-sectional study designs. The 2012 survey followed a PBPS protocol with random selection at village and household level with a comparable number of children sampled overall. The survey presented here sampled lower numbers of villages and households, but this is unlikely to sufficiently explain the large difference between the resultant TF prevalences found. Specific information on which villages were surveyed in the earlier study was not available, therefore it is not clear whether there was overlap between clusters visited; our randomisation process may have missed trachoma hotspots in Western Division by chance. The low estimate of Ct infection prevalence in this study does not support the estimated prevalence of TF observed in the previous survey, and could be a result of the relative non-specificity of phenotypic markers in trachoma.

The reported prevalence of TF and TT in Fiji varies significantly between studies. Tests for infection confirm that ocular Ct infections do still occur in Fiji, albeit infrequently and at relatively low load. Our clinical data suggest that trachoma does not meet the WHO definition of a public health problem in this Division of Fiji, but the inconsistency with previous studies warrants further investigation. It is also not clear whether the results from this division will be generalizable to the rest of the country. Recommendations on how best to incorporate this information into trachoma management plans are sparse. Estimating the age-specific prevalence of serological markers for exposure to chlamydial infection [37,38], photographic

evidence of phenotype, and exploration of other associations of TF in Fiji could be beneficial in developing those recommendations.

Supporting Information

S1 Table. Indicates the aspects of the study that adhered to the Strengthening the Reporting of Observational Studies in Epidemiology (STROBE) guidelines [39].
(PDF)

Author Contributions

Conceived and designed the experiments: CKM RB ER CHR MK DCWM AWS KN. Performed the experiments: CKM KN LC UM MK ER RB. Analyzed the data: CKM RB NA CHR AWS. Contributed reagents/materials/analysis tools: ALP RW. Wrote the paper: CKM RB CHR AWS DCWM. Critically reviewed the manuscript: CKM RB UM KN ALP RW NA DCWM ER MK LC CHR AWS.

References

1. World Health Organization. WHO Alliance for the Global Elimination of Blinding Trachoma by the year 2020: Progress report on elimination of trachoma, 2013. Wkly Epidemiol Rec. 2014; 96: 421–428.
2. West SK, Congdon N, Katala S, Mele L. Facial cleanliness and risk of trachoma in families. Arch Ophthalmol. 1991; 109: 855–7. Available: http://www.ncbi.nlm.nih.gov/pubmed/2043075 PMID: 2043075
3. Emerson PM, Lindsay SW, Alexander N, Bah M, Dibba S-M, Faal HB, et al. Role of flies and provision of latrines in trachoma control: cluster-randomised controlled trial. Lancet. 2004; 363: 1093–8. doi: 10.1016/S0140-6736(04)15891-1 PMID: 15064026
4. Thylefors B, Dawson CR, Jones BR, West SK, Taylor HR. A simple system for the assessment of trachoma and its complications. Bull World Health Organ. 1987; 65: 477–83. Available: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2491032&tool=pmcentrez&rendertype=abstract PMID: 3500800
5. Solomon AW, Peeling RW, Foster A, Mabey DCW. Diagnosis and assessment of trachoma. Clin Microbiol Rev. 2004; 17: 982–1011. doi: 10.1128/CMR.17.4.982–1011.2004 PMID: 15489358
6. Ngondi J, Reacher M, Matthews F, Brayne C, Emerson P. Trachoma survey methods: a literature review. Bull World Health Organ. 2009; 87: 143–51. Available: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2636192&tool=pmcentrez&rendertype=abstract PMID: 19274367
7. Solomon AW, Pavluck AL, Courtright P, Aboe A, Adamu L, Alemayehu W, et al. The Global Trachoma Mapping Project: Methodology of a 34-Country Population-Based Study. Ophthalmic Epidemiol. 2015; 22: 214–25. doi: 10.3109/09286586.2015.1037401 PMID: 26158580
8. Ward B. The prevalence of active trachoma in Fiji. Am J Ophthalmol. 1965; 59: 458–63. Available: http://www.ncbi.nlm.nih.gov/pubmed/14265582 PMID: 14265582
9. Swanston C. [Trachoma in the Fiji Islands]. Rev Int Trach. 1953; 30: 374–94. Available: http://www.ncbi.nlm.nih.gov/pubmed/13135064 PMID: 13135064
10. Stuppel R. Trachoma in Fiji—an original investigation. Br J Ophthalmol. 1933; 17: 88–97. Available: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=511514&tool=pmcentrez&rendertype=abstract PMID: 18169098
11. Maccallan AF. Trachoma in the British Colonial Empire—its relation to blindness, the existing means of relief, means of prophylaxis. Br J Ophthalmol. 1934; 18: 625–45. Available: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=511737&tool=pmcentrez&rendertype=abstract PMID: 18169234
12. Mathew AA, Keeffe JE, Le Mesurier RT, Taylor HR. Trachoma in the Pacific Islands: evidence from Trachoma Rapid Assessment. Br J Ophthalmol. 2009; 93: 866–70. doi: 10.1136/bjo.2008.151720 PMID: 19174394
13. Kama M, Cama A, Rawalai K, Koroivueta J. Active Ocular Trachoma In Fiji- A Population Based Prevalence Survey. Fiji J Public Heal. 2013; 2: 11–17.
14. Lees J, McCool J, Woodward A. Eye health outreach services in the Pacific Islands region: an updated profile. N Z Med J. 2015; 128: 25–33. Available: http://www.ncbi.nlm.nih.gov/pubmed/26367510
15. Fiji Bureau of Statistics. 2007 Census of Population. In: Suva, Fiji. 2007.

16. Solomon AW, Holland MJ, Burton MJ, West SK, Alexander NDE, Aguirre A, et al. Strategies for control of trachoma: observational study with quantitative PCR. Lancet. 2003; 362: 198–204. doi: 10.1016/S0140-6736(03)13909-8 PMID: 12885481

17. Solomon AW, Mohammed Z, Massae PA, Shao JF, Foster A, Mabey DCW, et al. Impact of mass distribution of azithromycin on the antibiotic susceptibilities of ocular Chlamydia trachomatis. Antimicrob Agents Chemother. 2005; 49: 4804–6. doi: 10.1128/AAC.49.11.4804–4806.2005 PMID: 16251338

18. Roberts CH, Last A, Molina-Gonzalez S, Cassama E, Butcher R, Nabicassa M, et al. Development and Evaluation of a Next-Generation Digital PCR Diagnostic Assay for Ocular Chlamydia trachomatis Infections. J Clin Microbiol. 2013; 51: 2195–203. doi: 10.1128/JCM.00622-13 PMID: 23637300

19. Last AR, Roberts CH, Cassama E, Nabicassa M, Molina-Gonzalez S, Burr SE, et al. Plasmid copy number and disease severity in naturally occurring ocular Chlamydia trachomatis infection. J Clin Microbiol. 2013; 52: 324. doi: 10.1128/JCM.02618-13 PMID: 24197878

20. Ripa T, Nilsson P. A variant of Chlamydia trachomatis with deletion in cryptic plasmid: implications for use of PCR diagnostic tests. Euro Surveill Eur Commun Dis Bull. France; 2006; 11: E061109.2.

21. R Core Team. R: A Language and Environment for Statistical Computing. In: R Foundation for Statistical Computing [Internet]. 2014. Available: http://www.r-project.org

22. Carpenter J, Bithell J. Bootstrap confidence intervals: when, which, what? A practical guide for medical statisticians. Stat Med. 2000; 19: 1141–64. PMID: 10797513

23. Louis TA. Confidence Intervals for a Binomial Parameter after Observing No Successes. Am Stat. Taylor & Francis Group; 1981; 35: 154.

24. Thein J, Zhao P, Liu H, Xu J, Jha H, Miao Y, et al. Does clinical diagnosis indicate ocular chlamydial infection in areas with a low prevalence of trachoma? Ophthalmic Epidemiol. 2002; 9: 263–9. Available: http://www.ncbi.nlm.nih.gov/pubmed/12187424 PMID: 12187424

25. Baral K, Osaki S, Shreshta B, Panta CR, Boulter A, Pang F, et al. Reliability of clinical diagnosis in identifying infectious trachoma in a low-prevalence area of Nepal. Bull World Health Organ. 1999; 77: 461–6. Available: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2557684&tool=pmcentrez&rendertype=abstract PMID: 10427930

26. Harding-Esch EM, Edwards T, Sillah A, Sarr I, Roberts CH, Snell P, et al. Active trachoma and ocular Chlamydia trachomatis infection in two Gambian regions: on course for elimination by 2020? PLoS Negl Trop Dis. 2009; 3: e573. doi: 10.1371/journal.pntd.0000573 PMID: 20027217

27. Burton MJ, Ramadhani A, Weiss HA, Hu V, Massae P, Burr SE, et al. Active trachoma is associated with increased conjunctival expression of IL17A and profibrotic cytokines. Infect Immun. 2011; 79: 4977–83. doi: 10.1128/IAI.05718-11 PMID: 21911461

28. Burr SE, Hart JD, Edwards T, Baldeh I, Bojang E, Harding-Esch EM, et al. Association between ocular bacterial carriage and follicular trachoma following mass azithromycin distribution in The Gambia. PLoS Negl Trop Dis. 2013; 7: e2347. doi: 10.1371/journal.pntd.0002347 PMID: 23936573

29. Schachter J. Will droplet digital PCR become the test of choice for detecting and quantifying ocular Chlamydia trachomatis infection? Maybe not. Expert Rev Mol Diagn. 2013; 13: 789–92. doi: 10.1586/14737159.2013.847792 PMID: 24134626

30. Roberts CH, Last A, Burr SE, Bailey RL, Mabey DC, Holland MJ. Will droplet digital PCR become the test of choice for detecting and quantifying ocular Chlamydia trachomatis infection? Maybe. Expert Rev Mol Diagn. 2014; 14: 253–6. doi: 10.1586/14737159.2014.897609 PMID: 24649815

31. Saftlas AF, Waldschmidt M, Logsden-Sackett N, Triche E, Field E. Optimizing buccal cell DNA yields in mothers and infants for human leukocyte antigen genotyping. Am J Epidemiol. 2004; 160: 77–84. doi: 10.1093/aje/kwh171 PMID: 15229120

32. Gaydos CA, Farshy C, Barnes M, Quinn N, Agreda P, Rivers CA, et al. Can mailed swab samples be dry-shipped for the detection of Chlamydia trachomatis, Neisseria gonorrhoeae, and Trichomonas vaginalis by nucleic acid amplification tests? Diagn Microbiol Infect Dis. Elsevier Inc.; 2012; 73: 16–20. doi: 10.1016/j.diagmicrobio.2012.02.008 PMID: 22578934

33. Morré SA, van Valkengoed IG, de Jong A, Boeke AJ, van Eijk JT, Meijer CJ, et al. Mailed, home-obtained urine specimens: a reliable screening approach for detecting asymptomatic Chlamydia trachomatis infections. J Clin Microbiol. 1999; 37: 976–80. Available: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=88635&tool=pmcentrez&rendertype=abstract PMID: 10074512

34. Dize L, Gaydos CA, Quinn TC, West SK. Stability of Chlamydia trachomatis on storage of dry swabs for accurate detection by nucleic acid amplification tests. J Clin Microbiol. 2014; 53: 1046–1047. doi: 10.1128/JCM.03218-14 PMID: 25540399

35. Last AR, Roberts CH, Burr SE, Cassama E, Nabicassa M, Mabey DCW, et al. Bacterial load in ocular Chlamydia trachomatis infection in a trachoma-hyperendemic population: geospatial analysis and

association with disease severity. Proceedings of the Thirteenth International Symposium on Human Chlamydial Infections. Pacific Grove, California; 2014. pp. 469–472.

36. International Association for the Prevention of Blindness. Trachoma Mapping in the Pacific. 2013.

37. Goodhew EB, Priest JW, Moss DM, Zhong G, Munoz B, Mkocha H, et al. CT694 and pgp3 as serological tools for monitoring trachoma programs. PLoS Negl Trop Dis. 2012; 6: e1873. doi: 10.1371/journal.pntd.0001873 PMID: 23133684

38. Martin DL, Bid R, Sandi F, Goodhew EB, Massae PA, Lasway A, et al. Serology for trachoma surveillance after cessation of mass drug administration. PLoS Negl Trop Dis. 2015; 9: e0003555. doi: 10.1371/journal.pntd.0003555 PMID: 25714363

39. von Elm E, Altman DG, Egger M, Pocock SJ, Gøtzsche PC, Vandenbroucke JP. The Strengthening the Reporting of Observational Studies in Epidemiology (STROBE) statement: guidelines for reporting observational studies. Lancet. 2007; 370: 1453–7. doi: 10.1016/S0140-6736(07)61602-X PMID: 18064739

CHAPTER 4: NOTES AND ADDITIONAL INFORMATION

4.2 Note A: Influence of storage temperature on longevity of *Ct* DNA

Typically, frozen storage is considered to be optimal for *Ct* specimen transport. Transport media is favored by commercial diagnostic manufacturers, but refrigerated storage of dry swabs does not affect diagnostic performance in the medium term (40 days) (300). Short-term dry storage at room temperature also does not affect diagnostic performance (301). In section 4.2, analysis of DNA recovery from synthetically prepared swabs suggests that storing swabs dry and refrigerated but unfrozen, fewer *Ct* targets were detected by ddPCR than from comparator swabs which were frozen. In the discussion, it is acknowledged that this may impact the detection of very low load specimens, and may result in an underestimate of infection prevalence. Synthetic swabs were prepared in the laboratory and stored under different conditions for up to 6 months to replicate this finding *in vitro*.

4.2.1 Methods

4.2.1.1 Suspension preparation

A suspension of cultured human epithelial type 2 (HEp-2) cells and serovar A *Ct* EBs in phosphate-buffered saline (PBS) was prepared to inoculate onto swabs. Stock HEp-2 cells were kindly provided by Dr Tamsyn Derrick (LSHTM). These were seeded into a 75 cm^2 culture flask containing Dulbecco's Modified Eagle Medium and grown until near-confluence. Cells were scraped from the flask and counted using a haemocytometer to determine the necessary dilution factor for spiking onto swabs. HEp-2 cells were spiked into PBS at approximately 400,000 per 1mL of PBS. Purified *Ct* A2497 EBs, kindly donated by Professor Martin Holland (LSHTM), were spiked into the same suspension at a dilution of 2μL of EBs per 1mL of PBS to achieve a high concentration of *Ct* and moderate concentration of *Homo sapiens* targets per PCR reaction.

4.2.1.2 Swab preparation

The suspension was sonicated and homogenized, and 50μL was aliquoted onto the head of polyester-coated swabs (Puritan Medical Products, Guilford, USA). Swabs were allocated to storage under one of three conditions: dry storage in polystyrene tubes at room temperature (uncontrolled, typically 22-25°C), dry storage in paper envelopes at room temperature inside a vacuum-sealed container with silica desiccant (in the field the purpose of this would be to ensure rapid and efficient desiccation of swab material and deter unwanted growth of microbial contaminants), or dry storage in polystyrene tubes at -20°C. Four swabs were prepared and processed per time point per storage condition. A single negative swab was prepared per time point per storage condition to control for contamination.

4.2.1.3 Swab processing

Swabs were removed from storage at 7, 30, 90 and 180 days and DNA was extracted using the swab protocol of the QIAamp DNA mini kit (Qiagen, Manchester, UK) according to manufacturer's instructions. Proprietary lysis buffer supplemented with 20 units of proteinase K was added directly to the swabs and incubated for 1 hour at 56°C. Lysate was bound to a silica spin column, washed and eluted into 100μL of 10mM Tris-Cl 1mM ethylenediaminetetraacetic acid (EDTA) buffer (TE). Swabs were tested using quantitative PCR. The primers and probes used are outlined in table 1 of the manuscript in section 4.2. Each 10-μL PCR reaction contained primers and probes at 300nM, 1x TaqMan Universal II Master Mix (Thermo Fisher Scientific, Waltham, USA) and 2μL of extracted DNA. Four technical replicates were tested per reaction.

4.2.1.4 Data analysis

Quantitation threshold and baseline parameters were set in SDS (version 2.4; Thermo Fisher Scientific, Waltham, USA). Exported quantitation cycle (C_q) data were analysed in R (302). Linear regression was used to determine whether the C_q decreased significantly with time in each individual treatment. The gradients of linear models were examined to determine whether there was a significant downward trend in load.

4.2.2 Results

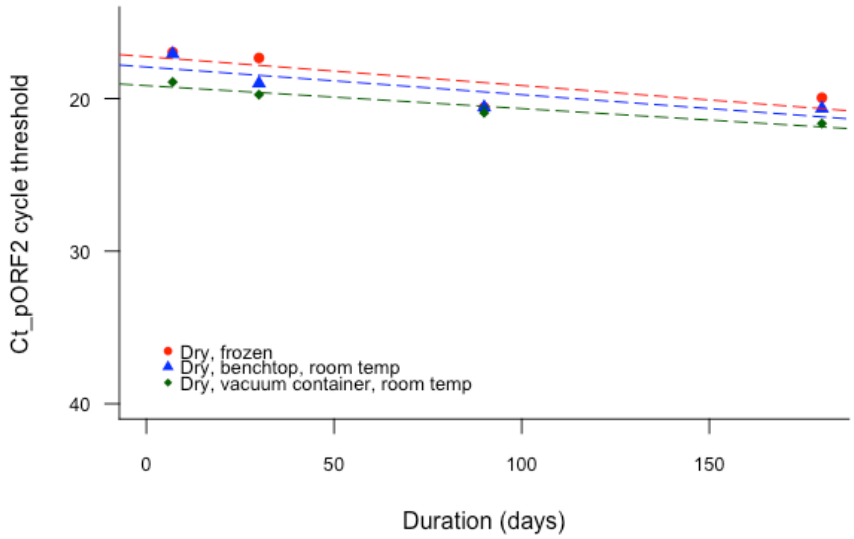
Ct and human DNA was readily detectable in all samples at all time points, suggesting no diagnostic impediment caused by 6 months storing high-load DNA at room temperature. All three treatments showed a significant increase in C_q required to detect *Ct* over 6 months according to linear regression models, indicating a decrease in target abundance. The gradient for each regression model had very subtle variations, but were broadly very similar. Based on these models the estimated rate of reduction in detectable load was 0.01–0.02% of the 7-day specimen C_q per day. After 6 months, the mean C_q for detection of plasmid had increased by 18% for the frozen swabs, and by 21% and 14%, respectively, for the desktop and vacuum contained room temperature swabs. The C_q for *omcB* target detection had increased by 9% for the frozen swabs, and by 17% and 14%, respectively, for the desktop and vacuum contained room temperature swabs. As C_q is an inverse exponential scale, these increases in approximately 1–3 cycles represent a decrease of between 70 and 90% of the material in real terms.

Table 4.2.2.1. Coefficients from linear regression models examining the relationship between cycle threshold and time in days.

Treatment	*Ct* pORF2		*Ct omcB*	
	Gradient	**p-value**	**Gradient**	**p-value**
Dry, frozen	0.019	< 0.0001	0.011	< 0.0001
Dry, desktop, room temperature	0.018	< 0.0001	0.018	< 0.0001
Dry, vacuum box, room temperature	0.015	< 0.0001	0.016	< 0.0001

Ct: Chlamydia trachomatis; pORF: Plasmid open reading frame; *omcB:* outer membrane protein complex B.

A



B

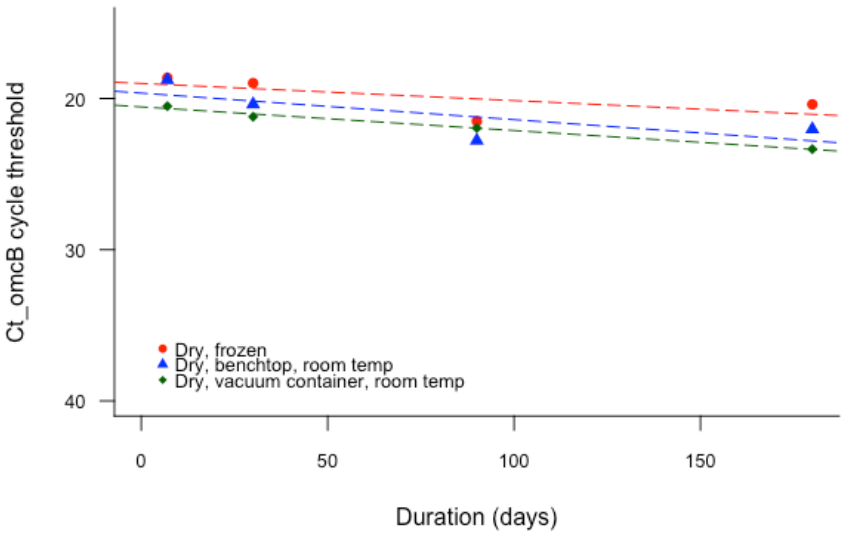


Figure 4.2.2.1. Change in recovered load of (A) *Chlamydia trachomatis* plasmid and (B) genomic targets following long-term storage frozen and at room temperature. Points represent mean of four swabs per time point per condition. Dashed lines represent linear regression model between load and time.

4.2.3 Discussion

These data demonstrate that there was a degradation of material with time for swabs stored dry, both when frozen and when stored at room temperature. Over prolonged periods, this loss may render the majority of the DNA undetectable. Intuitively, this might attributable to degradation of specimen DNA. However, due to the exquisite sensitivity of nucleic acid amplification techniques, this is only likely to lead to diagnostic failure in a minority of very low load specimens. The linear regression analysis suggests that the rate of degradation is consistent, regardless of storage temperature. This supports previous data showing that *Ct* DNA persisted for up to 2 years in a number of transport media, regardless of storage temperature (303). This finding is important for this study because it suggests that the reduced detection of targets in the field stored swabs described in the manuscript (299) may not have been due to more rapid degradation, but due to unequal spiking in the first instance. There was no pre-storage comparator group so it is not possible to further examine this possibility. However, the manuscript acknowledges that the *Ct* infection prevalence estimate may be an under-estimate because of the storage technique used, but *in vitro* data suggest that freezing the swabs would have led to a similar prevalence estimate. The data also suggest that long-term storage may lead to an under-estimate of prevalence if no transport media is used.

4.3 Trachoma in Fiji

From the findings presented in this chapter and subsequent studies, it seems most likely that the previous estimates of TT prevalence had somehow been inflated. Further research in to the trichiasis problem in Fiji was conducted by Macleod, Yalen and colleagues who undertook behavioral surveys to investigate the reasons for the inflated estimate during Kama and colleagues' survey. During a PBPS of epilating behaviors (304) (Appendix 3), approximately 7% of people in the Western Division of Fiji were found to epilate their eyelashes for cultural and social reasons not related to trachoma. Of 125 epilators, 124 did not have evidence of TS. This prevalence was remarkably similar to the rate of TT in the Western Division and authors identified that in the WHO simplified grading definition of trachoma, "evidence of recent epilation of in-turned lashes" could be applied to all epilated lashes, as it may not be clear to an examiner whether the epilated lash has been in-turned (304). Those cases were correctly identified as TT under the WHO simplified grading system, and the work has supported a revision of protocol for grading TT, whereby the eyelid must be everted where possible and TS observed before TT can be called.

Subsequent to the work that constitutes this paper, a serosurveillance study was undertaken in Fiji (305) (Appendix 2), which found that whilst 20.9% of children aged 1–14 years had antibodies to Pgp3, seropositivity did not significantly increase between the ages of 1 and 14 years, indicating little or no transmission of ocular *Ct* during childhood years. The seroprevalence to Pgp3 was significantly higher in iTaukei Fijians than in Indian Fijians. This could be attributable to the documented higher urogenital *Ct* prevalence in that group,

suggesting the Pgp3 exposure in children in Fiji may in part be due to urogenital exposure during parturition (305).

While data on epilation may provide a plausible explanation for the unusually high TT estimate, unanswered questions remain in Fiji. The prevalence of TF also varied markedly between this and previous studies. One limitation of the study in section 4.1, and a potential reason for the discrepancy between this and previous prevalence estimates, is the difference in sampling frame of the two studies. Kama *et al.* visited all divisions, and also may have included both Yasawa and Mamanuca Island groups in their sampling frame for the Western Division. Those living in the western part of Vitu Levu are likely to experience a different way of life to those in the outer island groups, and are likely to have better infrastructure and access to healthcare facilities. The outer islands may, therefore, be expected to have higher trachoma prevalence, although data to support this hypothesis are only anecdotal (A Cama, personal communication), and excluding them from the sampling frame may lead to an underestimate of Division-wide disease prevalence. The cluster distribution in Kama's study is not explicitly depicted in the manuscript, therefore we cannot estimate the effect of this beyond speculation. Additionally, during the study in section 4.1, a total of 1038 children were recruited into the study from 413 households, suggesting more 1–9 year olds per household than the national average (0.9 children aged 0–9 years per household listed in the 2007 National Census, compared to 2.5 children aged 1–9 years identified per household in this survey). There may be several explanations for this, for example, social practices in Viti Levu may involve communal living and may not be amenable to the definition of a household used in this survey, or larger households may have been selected by chance. Detailed data on household or family membership were not collected during the survey in section 4.1, therefore the effect of this difference on the study outcome cannot be quantified. In some settings, larger household size has been associated with increased risk of active trachoma (306) so selection of larger households may lead to overestimation of disease prevalence. Given the very low TF prevalence observed in our survey, this seems unlikely. However, the aim of the study was to measure the association between *Ct* infection and TF in Fiji and not to repeat the findings of the previous survey; these differences in study design between different studies did not significantly inhibit the capacity of the study to address that aim.

Our understanding of trachoma in Fiji has undoubtedly been advanced by these studies. However, the Fijian context has highlighted a much more important issue; namely that as the simplified trachoma grading system is rolled out around the world, confounding features may be identified which reduce the specificity of the system for identifying trachoma.

5. OCULAR *CHLAMYDIA TRACHOMATIS* INFECTION IN THE SOLOMON ISLANDS

5.1 Introduction

From the clinical data presented in chapter three, three patterns of disease were observed. In Kiribati, trachoma presents a significant public health problem. In Fiji, TF estimates differ between surveys, but recent data from the Western Division suggest trachoma not to be prevalent there. Vanuatu and the Solomon Islands both appeared to have prevalent TF, but no TT. The absence of TT led us to question the involvement of ocular *Ct* infection in this context. Temotu province in the Solomon Islands was suggested to have the highest TF prevalence by local collaborators, and was therefore selected as the most appropriate site to investigate the relationship between ocular *Ct* and TF. Due to the small size of Rennell & Bellona province, it was included in the EU.

The findings presented in this chapter generate subsequent lines of enquiry that eventually suggested that, although trachoma was present in the Solomon Islands, a substantial proportion of the clinical signs could not be attributed to ocular *Ct* infection.

5.2 Manuscript

In this manuscript, a combination of PBPS, test for infection and WGS are used to demonstrate that cases of TF, TI, TS, TT and *Ct* infection with ocular strains were found in the Solomon Islands, but that the level of *Ct* infection was lower than found in other populations with similar TF burden. Interestingly, the peak age-specific burden of trachoma was not in children under 5 years, as has been observed elsewhere (30), but apparently in 6 year-olds.

These data suggest that the use of clinical signs as a tool for guiding trachoma intervention might not be universally appropriate and that TF levels in Solomon Islands did not accurately reflect the population burden of ocular *Ct*. This topic will be revisited in more detail in chapters six and seven.

RESEARCH PAPER COVER SHEET

SECTION A – Student Details

Student	*Robert Butcher*
Principal Supervisor	*Chrissy h Roberts*
Thesis Title	*Using alternate indicators to define need for public health intervention for trachoma: Evidence from the Pacific Islands*

If the Research Paper has previously been published, please complete Section B, if not please move to Section C

SECTION B – Paper already published

Where was the work published?	*PLoS NTDs*		
When was the work published?	*September 2016*		
If the work was published prior to registration for your research degree, give a brief rationale for its inclusion	*NA*		
Have you retained copyright for the work?*	*Yes*	**Was the work subject to academic peer review?**	*Yes*

If yes, please attach evidence of retention. If no, or if the work is being included in its published format, please attach evidence of permission from the copyright holder (publisher or other author) to include this work.* ***Work published under Creative Commons Attribution 4.0 International Open Access License.*

SECTION C – Prepared for publication, but not yet published

Where is the work intended to be published?	*NA*
Please list the paper's authors in the intended authorship order:	*NA*
Stage of publication	*NA*

SECTION D – Multi-authored work

For multi-authored work, give full details of your role in the research included in the paper and in the preparation of the paper.	*I supported Anthony Solomon in securing funding for this project. The overall study was designed as part of the GTMP, I worked with Anthony to design and plan the infection components of the survey. I helped Oliver Sokana, Kelvin Jack, Eric Kalae and Leslie Sui to conduct the fieldwork and performed the laboratory work myself. I analysed the data with the help of Chrissy Roberts. I prepared the figures and tables, and wrote and revised the manuscript following feedback from co-authors.*

Student signature: _____ **Date:** 09/12/16

Supervisor signature: _____ **Date:** 19/12/16

PLOS | NEGLECTED TROPICAL DISEASES

RESEARCH ARTICLE

Low Prevalence of Conjunctival Infection with *Chlamydia trachomatis* in a Treatment-Naïve Trachoma-Endemic Region of the Solomon Islands

Robert M. R. Butcher[1]*, Oliver Sokana[2], Kelvin Jack[2], Colin K. Macleod[1,3], Michael E. Marks[1,4], Eric Kalae[5], Leslie Sui[5], Charles Russell[6], Helena J. Tutill[7], Rachel J. Williams[7], Judith Breuer[7], Rebecca Willis[8], Richard T. Le Mesurier[9], David C. W. Mabey[1,4], Anthony W. Solomon[1], Chrissy h. Roberts[1]

1 Clinical Research Department, Faculty of Infectious and Tropical Diseases, London School of Hygiene & Tropical Medicine, London, United Kingdom, **2** Eye Department, Solomon Islands Ministry of Health and Medical Services, Honiara, Solomon Islands, **3** Sightsavers, Haywards Heath, United Kingdom, **4** Hospital for Tropical Diseases, University College London Hospitals, London, United Kingdom, **5** Primary Care Department, Lata Hospital, Lata, Santa Cruz, Solomon Islands, **6** Bellona Rural Health Centre, Bellona, Solomon Islands, **7** Division of Infection & Immunity, Faculty of Medical Sciences, University College London, London, United Kingdom, **8** Task Force for Global Health, Decatur, Georgia, United States of America, **9** Centre for Eye Research Australia, University of Melbourne, Royal Victorian Eye and Ear Hospital, Melbourne, Australia

* robert.butcher@lshtm.ac.uk

OPEN ACCESS

Citation: Butcher RMR, Sokana O, Jack K, Macleod CK, Marks ME, Kalae E, et al. (2016) Low Prevalence of Conjunctival Infection with *Chlamydia trachomatis* in a Treatment-Naïve Trachoma-Endemic Region of the Solomon Islands. PLoS Negl Trop Dis 10(9): e0004863. doi:10.1371/journal.pntd.0004863

Editor: Joseph M. Vinetz, University of California San Diego School of Medicine, UNITED STATES

Received: February 2, 2016

Accepted: June 29, 2016

Published: September 7, 2016

Data Availability Statement: The clinical data for the manuscript are available in S1 Table. Sequence data used in phylogenetic analysis are available from GenBank. All chromosome sequences were mapped to the C. trachomatis A/HAR/13 reference sequence (GenBank accession number: CP000051.1) and plasmid sequences mapped to B/Jali/20 reference sequence (GenBank accession number: FM865436.1). The individual accession numbers for the bacterial isolate sequences are shown in S2 Table.

Abstract

Background

Trachoma is endemic in several Pacific Island states. Recent surveys across the Solomon Islands indicated that whilst trachomatous inflammation—follicular (TF) was present at levels warranting intervention, the prevalence of trachomatous trichiasis (TT) was low. We set out to determine the relationship between chlamydial infection and trachoma in this population.

Methods

We conducted a population-based trachoma prevalence survey of 3674 individuals from two Solomon Islands provinces. Participants were examined for clinical signs of trachoma. Conjunctival swabs were collected from all children aged 1–9 years. We tested swabs for *Chlamydia trachomatis* (*Ct*) DNA using droplet digital PCR. Chlamydial DNA from positive swabs was enriched and sequenced for use in phylogenetic analysis.

Results

We observed a moderate prevalence of TF in children aged 1–9 years (n = 296/1135, 26.1%) but low prevalence of trachomatous inflammation—intense (TI) (n = 2/1135, 0.2%) and current *Ct* infection (n = 13/1002, 1.3%) in children aged 1–9 years, and TT in those

Funding: The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript. AWS and RB were supported by the Wellcome Trust (098521/B/12/Z). CHR is supported by the Wellcome Trust Institutional Strategic Support Fund (105609/Z/14/Z). MM is supported by a Wellcome Trust Clinical Research Fellowship (102807/Z/13/Z) The fieldwork was jointly funded by the United Kingdom's Department for International Development Global Trachoma Mapping Project grant (ARIES: 203145) to Sightsavers, and by the Fred Hollows Foundation Australia (FHF 1041). Funding for the laboratory work was provided by the Fred Hollows Foundation Australia (FHF 1041). JB, HT and RJW are members of the PATHSEEK consortium, which is funded by the European Union's Seventh Programme for research, technological development and demonstration under grant agreement no. 304875. JB also receives funding from the NIHR UCL/UCLH Biomedical Research Centre.

Competing Interests: All authors declare that they have no conflicts of interest.

aged 15+ years (n = 2/2061, 0.1%). Ten of 13 (76.9%) cases of infection were in persons with TF or TI (p = 0.0005). Sequence analysis of the *Ct*-positive samples yielded 5/13 (38%) complete (>95% coverage of reference) genome sequences, and 8/13 complete plasmid sequences. Complete sequences all aligned most closely to ocular serovar reference strains.

Discussion

The low prevalence of TT, TI and *Ct* infection that we observed are incongruent with the high proportion of children exhibiting signs of TF. TF is present at levels that apparently warrant intervention, but the scarcity of other signs of trachoma indicates the phenotype is mild and may not pose a significant public health threat. Our data suggest that, whilst conjunctival *Ct* infection appears to be present in the region, it is present at levels that are unlikely to be the dominant driving force for TF in the population. This could be one reason for the low prevalence of TT observed during the study.

Author Summary

Trachoma is the most common infectious cause blindness worldwide, and the target of a global elimination initiative. A package of community-wide interventions is recommended to treat trachoma, which aim to reduce transmission of the causative agent *Chlamydia trachomatis*. These interventions require significant, prolonged investment. Clinical observation of follicles on the underside of the eyelid is used to assess requirement for and success of intervention. However, we now know that there are nonchlamydial causes of these follicles, so the observation of this clinical sign may not be specific for chlamydial infection. A recent study showed that infection testing can be cost-effective in low infection prevalence settings, as the money spent on infection testing is more than offset by the savings from avoiding additional rounds of community-wide interventions. We show here that, despite a high prevalence of clinical signs of disease, the estimated prevalence of chlamydial infection is low. The prevalence of the sight-threatening end stage of disease is also low, so we must consider whether the costly community-wide interventions are appropriate in this setting. The use of molecular tools at the population level to guide trachoma policy is still under investigation; this study will contribute to the pool of data required to assess the utility of these tools.

Introduction

Trachoma, caused by ocular strains of *Chlamydia trachomatis* (*Ct*), is the leading infectious cause of blindness worldwide [1]. Ocular infection with *Ct* is associated with a characteristic follicular conjunctivitis, known as "trachomatous inflammation—follicular" (TF) [2], which can persist for some time after the initiating infection has been cleared. In some individuals, infection can also cause the sign "trachomatous inflammation—intense" (TI). Repeated and prolonged bouts of severe inflammatory disease can lead to trachomatous scarring [3] (TS) which, in some individuals, can eventually cause the eyelashes to turn inwards, producing trachomatous trichiasis (TT), a condition in which the lashes painfully abrade the cornea. In combination with other trachoma-induced changes to the ocular surface, this may lead to corneal

opacity (CO) and blindness [2,4]. In 2014, the World Health Organization (WHO) estimated trachoma to be a public health problem in 51 countries, and responsible for approximately 2.2 million cases of visual impairment. Efforts to globally eliminate the disease as a public health problem are promising, with several countries having reported reaching elimination goals [1].

The island states of the Western Pacific Region are made up of several thousand widely dispersed volcanic islands and coral atolls. It has long been suspected that trachoma is endemic in these islands, with reports from the early twentieth century indicating that trachoma was present [5–9]. More recently, trachoma rapid assessments (TRA) conducted in the Pacific indicated the presence of trachoma in Fiji, Vanuatu, Solomon Islands, Nauru and Kiribati. Although TRAs do not give accurate estimates of disease prevalence, the TRA data suggested that, whilst TF levels appeared high, both TI and TT were surprisingly scarce [10].

A population-based prevalence survey (PBPS) is the gold standard for estimating district-wide trachoma burden [11] and trachoma PBPSs took place between 2011 and 2014 in some districts of the Solomon Islands, Kiribati and Fiji [12]. They reported that TF prevalence was above the WHO-recommended threshold (≥10% TF in children aged 1–9 years) for public health interventions in the Melanesian-dominated districts studied (Solomon Islands and Fiji). Those surveys also reported surprisingly low levels of TT [12,13] (at or below the WHO elimination threshold of 0.1% in the all-ages population[14]) compared to those observed in other populations with highly prevalent TF [15,16].

The seemingly discrepant (with respect to out-of-region comparators) finding of high prevalence of TF in populations with negligible levels of TI or TT led us to question whether ocular *Ct* infections are present in Melanesia. We augmented GTMP mapping of Temotu and Rennell and Bellona provinces in the Solomon Islands, with tests for infection and next-generation sequencing, to determine the prevalence of *Ct*, and whether *Ct* and active trachoma were associated. If *Ct* was detected in conjunctival specimens, we considered whether those strains were of an ocular or genital genotype. We considered how our results compare to other published datasets.

Methods

Ethics statement

The study adhered to the tenets of the Declaration of Helsinki. The London School of Hygiene & Tropical Medicine Ethics Committee (6319 and 6360) and the Solomon Islands National Health Research and Ethics Committee (HRC13/18) granted ethical approval for this study.

Village and household heads were consulted prior to enrolment. Individuals were informed of the nature and requirements of the study prior to enrolment, by a staff member fluent in local dialects, and were asked to provide written evidence of consent. For those aged 18 years and under, written consent of a parent or guardian was required.

Study design

Our study was conducted alongside GTMP survey teams as they undertook mapping of one evaluation unit (EU) in the Solomon Islands in October and November, 2013. In the GTMP study, an EU was defined as a single administrative province, however, Temotu and Rennell and Bellona were grouped together into a single EU due to their small populations. Local healthcare workers identified Temotu and Rennell and Bellona as the provinces with the highest suspected trachoma burden in the country. The study was a cross-sectional cluster-randomised PBPS of trachoma. We determined *a priori* that 1019 children aged 1–9 years should be sampled to estimate infection prevalence of 10% with a precision of ±3% at the 95% confidence

level, assuming a design effect of 2.65 [17]. Our sample size was framed around the population of children in this age range, as they are the group most likely to harbour infection [15,18].

The Land Registry listed 533 villages across the two provinces in 2013. Local healthcare workers identified villages that were not currently inhabited, and remaining villages were eligible for simple random selection. In each cluster a targeted number of households were randomly selected from a full list of village households to recruit the required number of children. All household residents over the age of 1 year in each selected household were eligible for inclusion [17].

Clinical examination and photography

Clinical examination was conducted by graders who had been certified according to GTMP protocols [17]. Clinical grading was carried out using the WHO simplified system, in which trichiasis is defined as at least one eyelash in contact with the eyeball (or evidence of recent removal of in-turned eyelashes), TF is defined as 5 or more follicles of >0.5mm diameter on the central part of the upper tarsal conjunctiva, and TI is defined as pronounced inflammatory thickening of the upper tarsal conjunctiva obscuring >50% of the deep tarsal vessels [2].

Conjunctival photographs were taken using a Nikon D3000 SLR camera and graded by an independent photograder who had previously had a kappa agreement score in excess of 0.9 when grading photographs also graded by a master grader. The photograder was masked to the corresponding field grade for each photograph.

Clinical sample collection, handling and processing

Conjunctival swabs were collected from all children aged 1–9 years. Polyester-coated cotton swabs (Puritan Medical Products, Guilford, ME, USA) were passed three times over the right tarsal conjunctiva with a 120° turn in between each pass [19–21]. The examiner changed their gloves between participants to avoid cross contamination in the field. 1 in 30 swabs did not touch the conjunctiva but were passed within 15 cm of a seated participant then stored and processed in identical fashion to other study specimens, to act as field contamination controls. Swabs were stored immediately in RNAlater (Life Technologies, Paisley, UK), kept on ice packs in the field before short-term storage at 4°C, and frozen within 48 hours of collection [22]. Specimens were transported to the UK on dry ice where they were stored at -80°C until they were extracted with Qiagen AllPrep DNA/RNA mini kits (Qiagen, Manchester, UK) according to manufacturer's recommendations.

Note A - page 107

Droplet digital PCR

DNA specimens were tested for the *Ct* plasmid using a droplet digital PCR assay targeting a single *Ct* plasmid target in duplex with a human ribonuclease gene, which acted as endogenous control. The published assay methodology was used [23] with minor protocol adjustments to the tested sample volume (4.95μL increased to 8μL) and oligonucleotide concentrations (primer concentration increased from 300nM to 900nM; probe concentration decreased from 300nM to 200nM). Samples were considered valid if there was >95% confidence in non-zero endogenous control concentration, and positive according to published criteria (>95% confidence in non-zero chlamydial plasmid load [23]). Samples from children with TF were retested with an alternative, quantitative assay targeting both chlamydial chromosomal and plasmid targets [24]. The oligonucleotides used are shown in Table 1.

Table 1. Oligonucleotides used in this study. Chlamydial targets adapted for ddPCR [23,24] based on sequences from Pickett and colleagues [87]. Endogenous control target adapted from Luo and colleagues [88].

Target	Oligo	Sequence (5′ to 3′)
Chlamydia trachomatis omcB	Primer (F)	GACACCAAAGCGAAAGACAACAC
	Primer (R)	ACTCATGAACCGGAGCAACCT
	Probe	FAM**-CCACAGCAAAGAGACTCCCGTAGACCG-BHQ1
Chlamydia trachomatis plasmid ORF2	Primer (F)	CAGCTTGTAGTCCTGCTTGAGAGA
	Primer (R)	CAAGAGTACATCGGTCAACGAAGA
	Probe	FAM*/HEX**-CCCCACCATTTTTCCGGAGCGA-BHQ1
Homo sapiens RPP30	Primer (F)	AGATTTGGACCTGCGAGCG
	Primer (R)	GAGCGGCTGTCTCCACAAGT
	Probe	HEX*-TTCTGACCTGAAGGCTCTGCGCG-BHQ1

F: forward; R: reverse; BHQ: Black Hole Quencher; omcB: outer membrane protein complex B; ORF: open reading frame; RPP30: ribonuclease P/MRP 30kDa subunit

*Diagnostic assay [23]

**Quantitative assay [24]

doi:10.1371/journal.pntd.0004863.t001

Chlamydial genome and plasmid sequencing

Chlamydial DNA was preferentially enriched in clinical samples using custom *Ct*-specific RNA baits in a SureSelect system, as developed by the PATHSEEK consortium. Due to the low biomass of conjunctival swabs, human carrier DNA was added to achieve the required DNA input concentration for sequencing. Samples were sequenced on the Illumina MiSeq platform [25].

Literature search

Articles were identified through two specific literature searches to illustrate how our data compared to existing published studies. PubMed hits, references contained within those articles and relevant articles from the authors' own archives were considered eligible for inclusion. Using the terms "population-based" and "trachoma", we identified population-level studies where TF had been reported in children aged 1–9 years and TT had been reported in adults over the age of 15 years in the same population. District-level data were extracted where more than a single district was reported on in a single publication. Using the search terms "trachoma" and "infection", we identified studies reporting population-level nucleic acid-based infection data alongside clinical data on TF (with or without TI). Data from communities who had received one or more rounds of MDA were excluded. During both literature searches, studies where age-specific trachoma prevalence in 1- or 2-year age bands was presented were also reviewed.

Data recording and analysis

Field data were recorded using a purpose-built Open Data Kit (https://opendatakit.org/) app [17]. Age adjustments were carried out using 5-year census age bands [26]. All analyses were carried out using R version 3.2.2 [27]. The overall agreement between field exam and photographic grade was determined using kappa agreement scores. The relationship between TF and *Ct* infection was tested using logistic regression. *Ct* loads in children with and without TF were compared using the Mann Whitney U test.

Following preliminary assessment of mapping quality to various *Ct* strains, 251-bp paired end reads were trimmed and mapped to *Ct* reference strain AHAR-13 genome sequence (GenBank accession CP000051.1) and B/Jali20 plasmid genome sequence (FM865436.1) using

Bowtie 2 [28]. SAMtools and BCFtools were used to index and assemble reads, and bases were called by collapsing reads vertically [29]. Trimmed reads were also mapped to E/Bour (genome HE601870.1, plasmid HE603212.1) to determine whether the choice of reference influenced branching points in the phylogram.

Consensus sequences were submitted for megablast search on National Centre for Biotechnology Information (NCBI) GenBank to determine nearest relatives based on genetic sequence. Whole genomes were aligned with progressiveMauve [30]. A core alignment was generated by extraction and amalgamation of locally collinear blocks using stripSubsetLCBs [31]. Distance matrices and bootstrapped phylogeny was inferred using phangorn, ape and SeqinR packages in R [32–34]. Regions orthologous to *ompA*, *trpA* and the plasticity zone (PZ; a ~20 kb region of the *Ct* chromosome between *dsbB* and *ycfV* [35]) were analysed in isolation due to their disproportionately high variability and influence on pathogenicity compared to the rest of the *Ct* chromosome. These were extracted from consensus sequences using BLASTn in the NCBI BLAST+ suite, and aligned using MUSCLE alignment software [36].

Results

Enrolment and disease prevalence

Note B - page 110

The combined population of Temotu (21,362), and Rennell and Bellona (3041) comprises 4.7% (24,403/515,870) of the total population of the Solomon Islands, according to the 2009 national census [26]. We surveyed 959 households in 32 clusters throughout this EU. 4049 people were enumerated, and 3674 (91%) consented to participate (a total of 17% of the provincial population, average 4.2 people per household). Data was not collected on the reasons people did not take part. The examined population included 1135 children aged 1–9 years (53% male), and 2061 adults aged 15 years and over (42% male). The median age was 18 years (min 1, max 100, inter-quartile range [IQR]: 8–38 years). In this population there were 397 (10.9%) cases of TF, 5 (0.1%) cases of TI, and 2 (0.1%) cases of trichiasis in either eye of subjects of all ages identified by field grading. 84% of cases of TF were bilateral.

In children aged 1–9 years, the prevalence of active trachoma (defined as presence of TF and/or TI in either eye) was 26.3% (TF: 26.1% [296/1135]; TI: 0.2% [2/1135]). The proportion of males in this age group with active trachoma was significantly higher than that of females in the same age group (28.9% [176/608] versus 23.1% [122/527], p = 0.027). When adjusted for age and sex, the prevalence of active trachoma was 22% (95% confidence interval [CI]: 18.5–26.0%). In adults aged 15 years and over, the prevalence of active trachoma was 1.2% (TF: 1% [21/2061]; TI: 0.1% [3/2061]). The prevalence of trichiasis in adults was 0.1% (2/2061). When adjusted for age and sex, the prevalence of TT was 0.04% (95% CI: 0–0.3%). The field team did not recall one case of trichiasis and the other was documented as mild, with a single lash contacting the globe away from the cornea.

Photographs were taken from 3110/3674 (85%) of participants. Preliminary quality control yielded 2418 (78%) photographs that were unsuitable for grading due to quality issues. A total of 692 photographs from study participants of all ages (238 in children aged 1–9 years) were evaluated by an independent grader. Photo-grading according to the simplified grading system agreed with TF in 94% of cases leading to Fleiss' kappa agreement scores of 0.88, which indicated excellent agreement between photo grader and field grading in TF calls. Cases of TS were noticed in the photo set, although the evidence was insufficient to determine the population prevalence of this sign.

Fig 1 shows exemplars of both mild and more severe TF in Solomon Island children, as well as a normal conjunctiva for contrast. The age-specific prevalence of TF cases is shown in Fig 2.

Fig 1. Photographs of conjunctivae showing (**A**) no evidence of active trachoma, (**B**) mild trachomatous inflammation-follicular (TF) and (**C**) more severe TF. All three photographs were taken of conjunctival *C*. *trachomatis* infection-negative children aged 1–9 resident in Temotu, Rennell or Bellona, Solomon Islands, October-November 2013, in whom the photo grade and field grade matched.

doi:10.1371/journal.pntd.0004863.g001

Conjunctival infection

Swabs were collected from 1076/1135 (94.8%) child participants aged 1–9 years, along with 41 field controls. Of those, 1002 (93.1%) passed quality control by testing positive for the

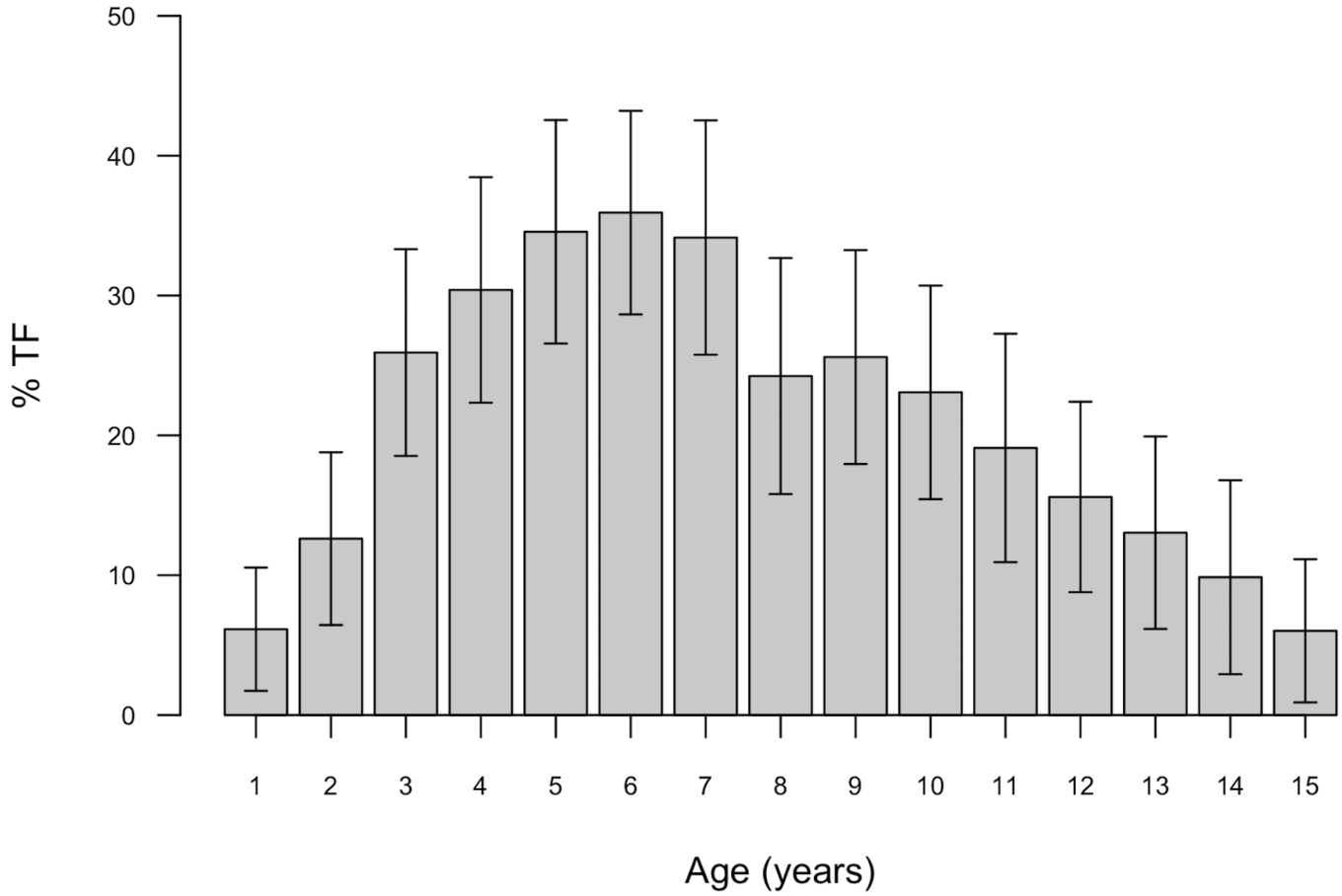


Fig 2. Age-specific prevalence (grey bars) and 95% confidence interval (arrows) of trachomatous inflammation—follicular (TF) in individuals aged 1–15 years, recorded during a trachoma survey of Temotu, Rennell and Bellona, Solomon Islands, October-November 2013.

doi:10.1371/journal.pntd.0004863.g002

endogenous control gene *H. sapiens RPP30*. All blank field controls and all known-negative extraction and PCR controls tested negative for endogenous control and microbial targets. Evidence of *Ct* infection was found in 13 (1.3%) of 1002 specimens. Of those who tested positive for plasmid on diagnostic screen, 9 also tested positive for *Ct* chromosomal target *omcB*.

Of the individuals whose swab was positive for the endogenous control *RPP30*, 257/1002 (25.7%) had TF and/or TI in the right eye (Table 2). The prevalence of *Ct* infection was 10/257 (3.9%) in those with TF and/or TI, and 3/745 (0.4%) in those without. Active disease status was highly significantly associated with current infection (odds ratio: 10.0, p = 0.0005).

While TF was observed in all 32 villages that were surveyed, we observed *Ct* infection in just eight villages. While the study was not designed to detect sub-EU-level differences in prevalence, *post hoc* analysis indicated there were significantly more cases of infection per capita in Rennell and Bellona than in Temotu province (6/131 [4.6%] versus 7/871 [0.8%], respectively; Mann Whitney U: p = 0.0004). TF levels in the 1–9 year old indicator group were in excess of 10% in both provinces included in the EU, but significantly lower in Rennell and Bellona (27.3% in Temotu versus 17.2% in Rennell and Bellona; Mann Whitney U: p = 0.0001).

Load of infection

The mean load of endogenous control target was 12,560 copies/swab (IQR: 872–13,980 copies/swab). In positive swabs, the median load of *Ct* plasmid was 13,840 copies/swab (IQR: 3599–84,990 copies/swab). There was a large difference in median load between *Ct* positive samples from children with active disease when compared to those without active disease, although the difference was not statistically significant (median 13,840 versus 782 copies/swab; Mann Whitney U test p = 0.81). The median *omcB* load was 7725 copies/swab (IQR: 1696–22,110 copies/swab) and the mean plasmid:genome ratio (i.e., plasmid copy number per bacterium) was 4.4 (IQR: 3.7–5.6) which is similar to that described elsewhere [24].

Genome sequencing

Note C - page 111

Sequencing was successful in 11/13 strains. The mean number of paired reads per specimen was 2.3 million (IQR: 2.1–2.7 million; Table 3). The median percentage of reads mapping to A/HAR-13 reference genome was 10.1% (IQR: 1.5–24.4%) per specimen. The median percentage of reads mapping to B/Jali20/OT reference plasmid was 2.1% (IQR: 0.3–4.8%) per specimen.

Complete genome sequences (>95% coverage) were obtained from five of 13 specimens, whilst partial genome sequences (<95% coverage) were obtained from six specimens. Complete (>95%) or partial (<95%) plasmid sequences were obtained from eight and three specimens, respectively. One specimen failed to sequence. The median coverage of at least 1× read depth of the reference genome was 51.5% (IQR: 12.4–98.2%); the median coverage of at least 1× of the reference plasmid was 99% (IQR: 61.4–99.7%).

Table 2. Frequency of conjunctival *C. trachomatis* infection and active trachoma during a trachoma survey in Temotu, Rennell and Bellona, Solomon Islands, October-November 2013.

ddPCR result	No TF/TI (%)	TF/TI (%)	*Total*
Positive	3 (0.4)	10 (3.9)	**13 (1.3)**
Negative	742 (99.6)	247 (96.1)	**989 (98.7)**
Total	**745**	**257**	**1002 (100)**

ddPCR: droplet digital polymerase chain reaction; TF: trachomatous inflammation—follicular; TI: trachomatous inflammation—intense.

doi:10.1371/journal.pntd.0004863.t002

Table 3. Sequencing parameters and results of BLASTn analysis of consensus sequence. Conjunctival *C. trachomatis* genomes obtained from Temotu, Rennell and Bellona, Solomon Islands, in October-November 2013.

Sample	Genetic component	Total paired reads	Number of paired reads mapped to reference (%)	% reference covered*	Serovar of closest NCBI BLASTn match
SB000209	Genome	1,948,968	445 (0.02)	0.2	F
	Plasmid		173 (0.01)	15.5	D
SB002563	Genome	1,838,502	513 (< 0.01)	0.5	I
	Plasmid		35 (< 0.01)	12.4	F
SB002739	Genome	2,530,443	403,672 (16.0)	95.8	A
	Plasmid		61,345 (2.4)	99.7	B
SB006908	Genome	2,594,512	34,663 (1.3)	18.5	J
	Plasmid		3857 (0.1)	76.7	D
SB006930	Genome	2,630,055	1,743,718 (66.3)	98.9	A
	Plasmid		293,102 (11.1)	99.8	B
SB008107	Genome	2,269,697	485,840 (21.4)	98.0	A
	Plasmid		79,314 (3.5)	99.8	B
SB011363	Genome	3,878,711	75,326 (1.9)	16.3	I
	Plasmid		17,375 (0.4)	99.2	B
SB011759	Genome	3,044,398	52,165 (1.7)	16.3	A
	Plasmid		11,707 (0.4)	96.0	B
SB011836	Genome	313	0 (0)	0.0	-
	Plasmid		0 (0)	0.0	-
SB012441	Genome	2,996,613	301,482 (10.1)	84.5	A
	Plasmid		63,827 (2.1)	99.5	B
SB013112	Genome	2,274,168	1,345,864 (59.2)	98.9	A
	Plasmid		332,462 (14.6)	99.8	B
SB013321	Genome	2,128,740	584,834 (27.5)	98.8	A
	Plasmid		127,976 (6.0)	98.8	B

* At least 1× read depth

doi:10.1371/journal.pntd.0004863.t003

BLASTn analysis of five complete Solomon Islands *Ct* consensus genome sequences against archived *Ct* genome sequences revealed that all five had closest sequence homology and alignment to serovar A type ocular strains. BLASTn analysis of eight complete Solomon Islands plasmid consensus sequences revealed highest sequence homology with published serovar B ocular strain plasmids.

Phylogenetic analysis of the complete Solomon Islands *Ct* genomes showed that these samples formed a single clade of closely related genotypes that formed a sub-clade of the ocular strains. The five complete genomes were more closely related to each other than they were to the nearest reference neighbour (A/HAR-13). Strains from the Solomon Islands were most closely related to the ocular serovars in the T2 chlamydial clade, as shown in Fig 3. Bootstrapping supported our phylogram by indicating resampling did not alter the branch position in >80% of bootstrap runs.

Outer membrane protein A (*ompA*) sequences from the ocular reference strains do not cluster in a single clade, as has been described previously [37]. *OmpA* orthologs from the five Solomon Island sequences were more closely related to each other than to their nearest neighbour which was the C-TW3 strain. Their relationship to other reference sequences is shown in S2A Fig. Additionally, tryptophan synthase alpha subunit (*trpA*) orthologs in Solomon Island sequences were most similar to ocular strains (S2B Fig) and featured a single nucleotide

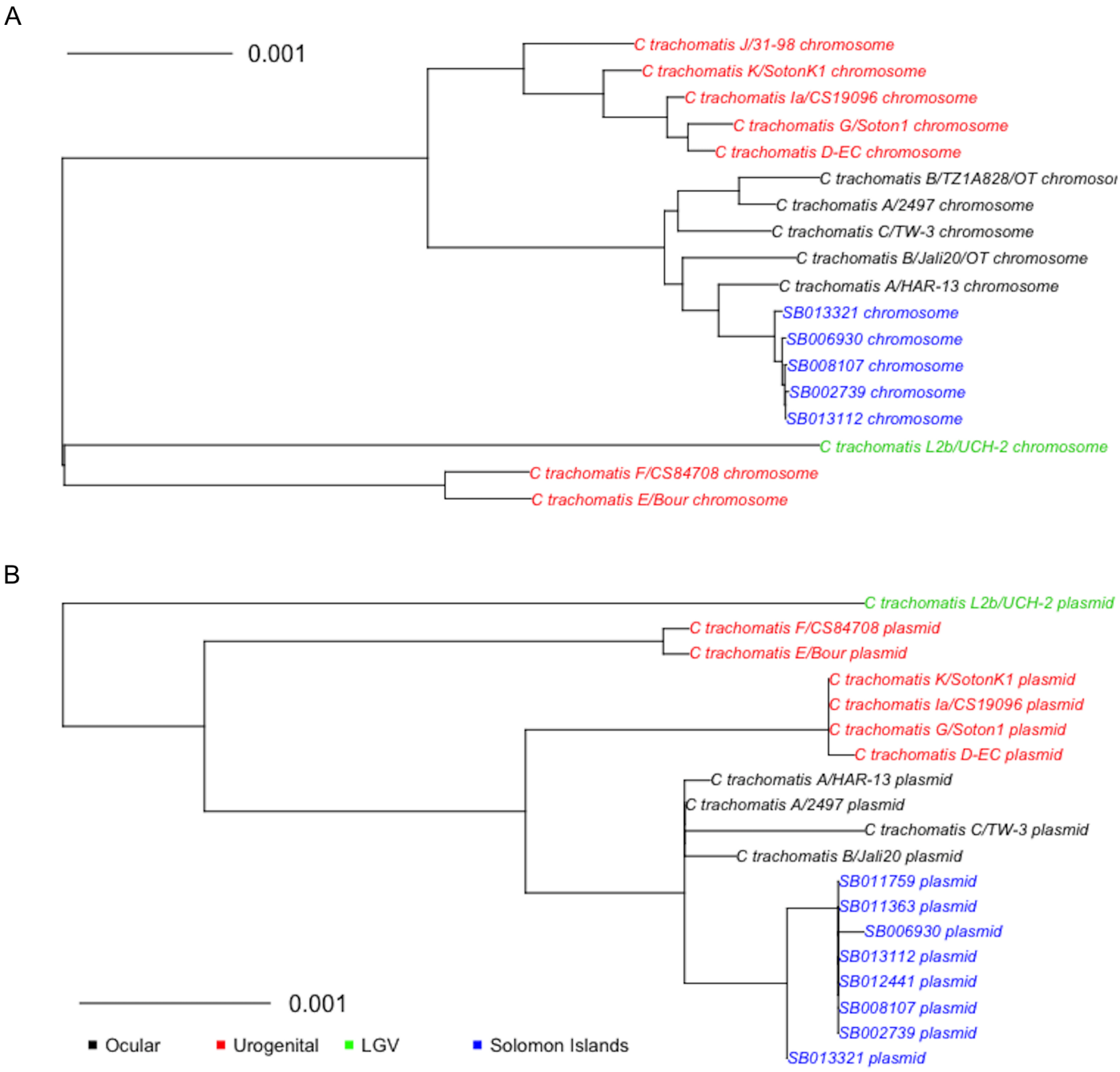


Fig 3. Maximum likelihood phylogram of (**A**) genome and (**B**) plasmid sequences from clinical specimens collected in the Solomon Islands in October and November 2013, assembled to *C. trachomatis* A/HAR-13 and B/Jali20/OT reference, respectively. All branches had bootstrap values over 85/100.

doi:10.1371/journal.pntd.0004863.g003

deletion leading to a premature stop codon and truncation of the open reading frame when compared to urogenital sequences. Finally, the relationship between the plasticity zone (PZ) of Solomon Island sequences compared to references was evaluated and is shown in supplementary Fig 2C.

Literature search

A low prevalence of TT has been observed in other populations in which TF was highly prevalent. Fig 4 shows data taken from published PBPSs in Nigeria [38–44], Niger [45], Sudan [46], Kenya [16], Ethiopia [47] and Cameroon [48,49] comparing the prevalence of TF in those aged 1–9 years with the prevalence of TT in 15+ year-olds in the same EUs, all of which were treatment-naïve. Of 58 identified EUs with comparable prevalence of TF in the 1–9 year olds (10% < TF < 40%) to that observed in the current study, 50% had a TT prevalence greater than 1% in those over the age of 15 years; the median TT prevalence was 1%, compared to 0.1% in our survey. Our population had a high TF prevalence when compared to other districts in this analysis with <1% TT.

Published data from studies in Ethiopia [50], Niger [50], Tanzania [18], Gambia [18], Cameroon [48], Mali [51] and Brazil [52] indicate that in other trachoma-endemic populations, the highest age-specific TF prevalence is generally in those aged 3–4 years. When published age-specific TF profiles from other parts of the world are compared to the age distribution in this study (Fig 5), the peak age-specific TF prevalence in our data is shown to be in an older age group.

We identified a number of previous studies have reported concomitant *Ct* infection and active trachoma prevalence estimates [19–21,53–70] in children aged 0–9 years or a subset of that group in 35 districts. At the population level there is a good correlation between the two (R = 0.84) (Fig 6). Our *Ct* infection estimate does not conform to the patterns observed in those other populations, with infection being substantially less prevalent than might be expected given the TF prevalence.

Discussion

We report an apparently mild trachoma phenotype in which TF is moderately prevalent yet *Ct* infection, TI and TT are rare. The findings reflect those of other studies; over 2300 adults were

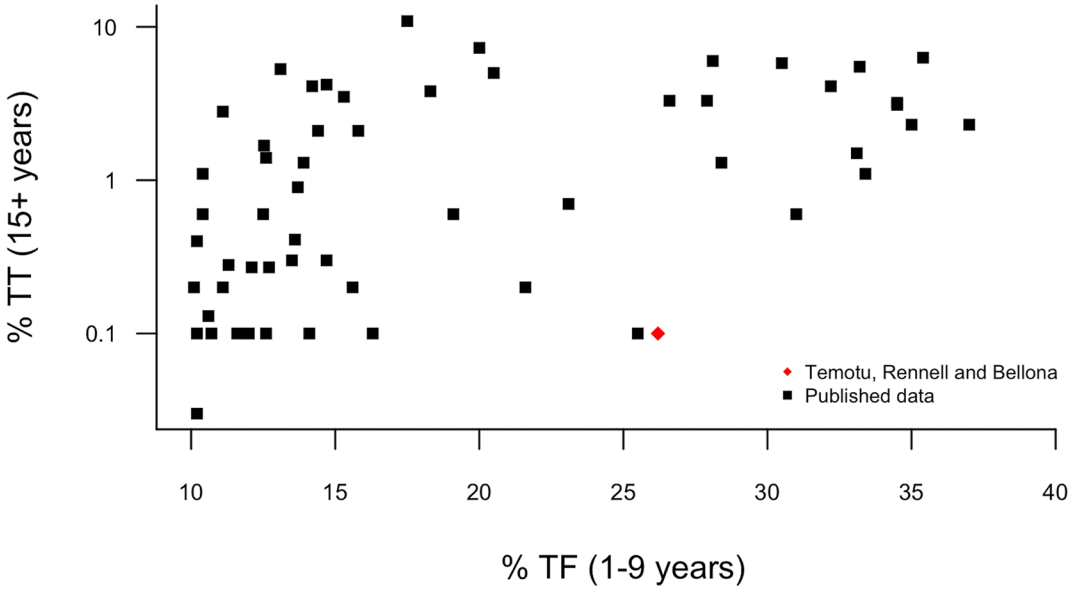


Fig 4. Comparison of unadjusted prevalence of trachomatous trichiasis (TT) in 15+ year olds, and trachomatous inflammation—follicular (TF) in 1–9 year-olds, in treatment-naïve trachoma-endemic EUs for which data have previously been published (n = 58), and in which the TF prevalence in 1–9 year-olds was 10–40%. Correlation coefficient (R) is 0.40 for this subset, but 0.77 if including studies from areas of any prevalence.

doi:10.1371/journal.pntd.0004863.g004

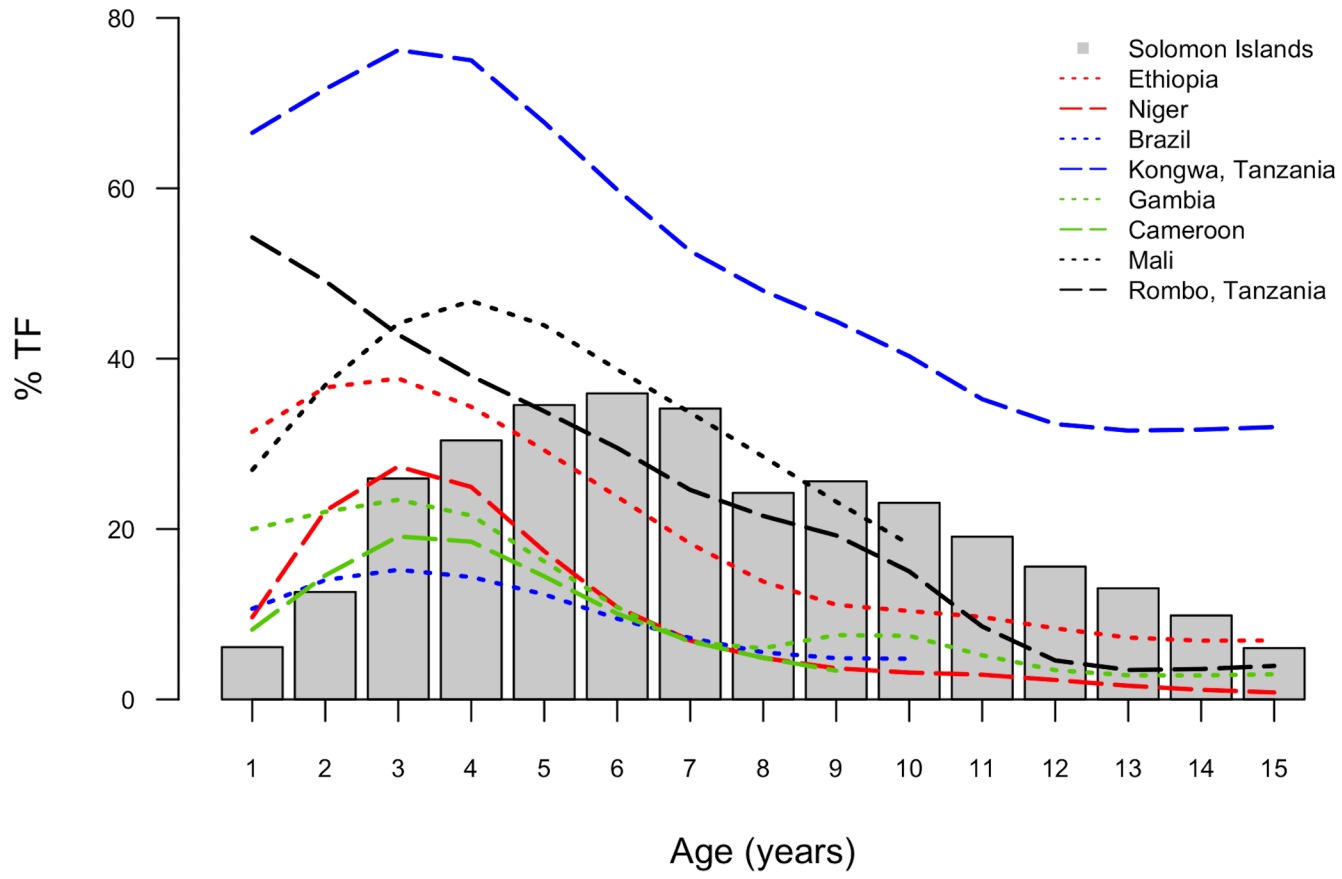


Fig 5. Published age-specific trachomatous inflammation—follicular (TF) prevalence data in studies undertaken in districts with >10% TF in overall child population (dotted and dashed lines), compared to the same in Temotu, Rennell and Bellona, Solomon Islands, October-November 2013 (grey columns).

doi:10.1371/journal.pntd.0004863.g005

surveyed in Makira, Isabel and Central provinces yet only 3 cases of TT identified, indicating an unadjusted prevalence among those adults of 0.1% despite 22.2% of the 1–9 years population in the same survey having signs of TF [12]. The TF prevalence in Temotu, Rennell and Bellona is the highest of the populations surveyed during GTMP mapping. Choiseul province had low levels of both TF (6%) and TT (0%). Interestingly, Western Province had more TT cases than Temotu, Rennell and Bellona [71]. Further studies are warranted to determine whether infection prevalence is also higher in that region. The prevalence of TF in these communities qualifies this EU for priority implementation of the A, F and E components of the SAFE strategy (**s**urgery to treat TT, mass **a**ntibiotic distribution to treat infection, promotion of **f**acial cleanliness and **e**nvironmental improvement to reduce transmission [72]), but the trichiasis data suggest the elimination target for TT has already been met. The evidence of this survey suggests that prevalence of TF in children may not be an appropriate marker of disease burden in this setting. Prolonged infection with *Ct*, intense transmission of *Ct*, presence of TI and other markers of inflammation have been associated with progression to TS [3,73–75], the precursor of TT. It is therefore feasible that the low prevalence of TI and *Ct* are related to the paucity of TT in this population.

In Fig 4 we demonstrate that the correlation between reported TF and TT prevalence is weak, suggesting that high TF prevalence is not always indicative of a significant TT burden. This is not an unexpected finding; the signs of TF are transient, being instigated and cleared

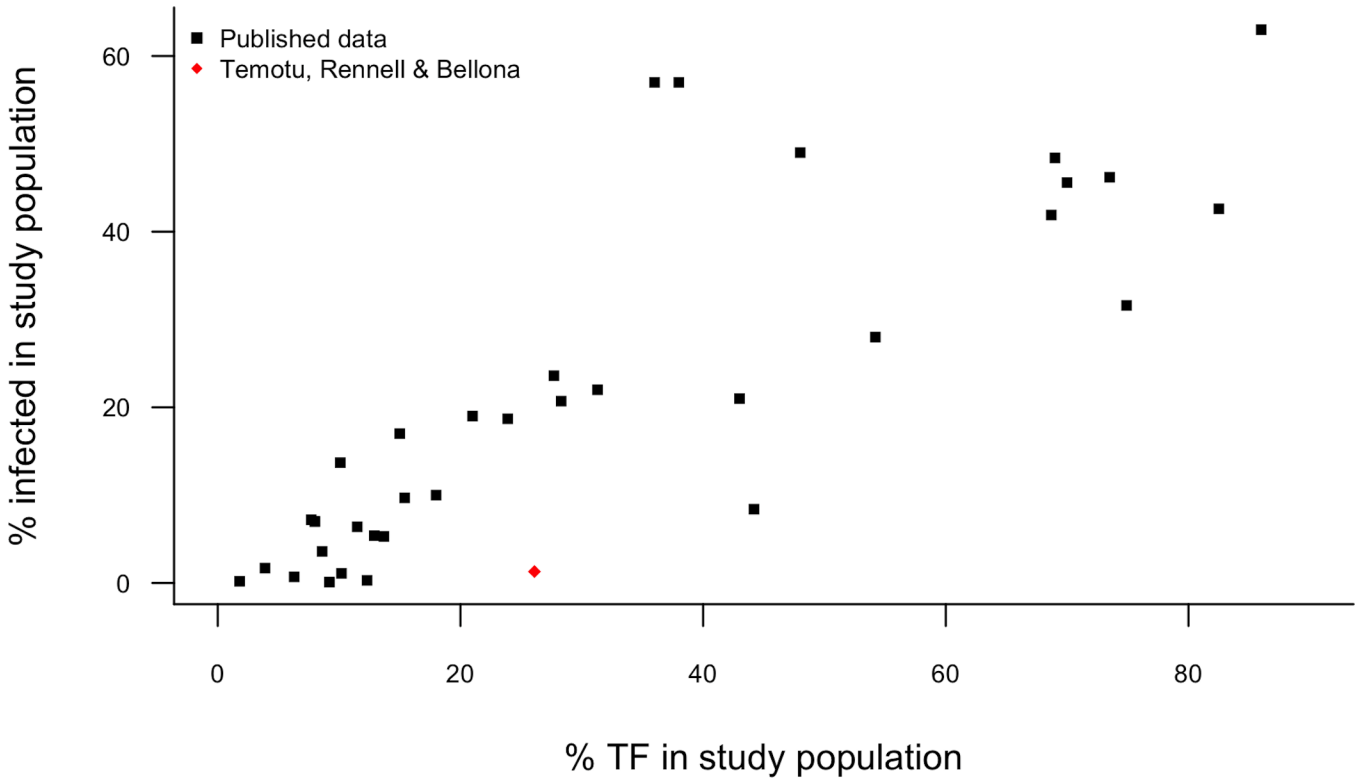


Fig 6. Relationship between the prevalence of conjunctival *C. trachomatis* infection (diagnosed by nucleic acid amplification test) and trachomatous inflammation—follicular (TF) in the total 0–9 year-old population or a subset of that group at the district level (n = 35 districts). Correlation coefficient (R) is 0.84.

doi:10.1371/journal.pntd.0004863.g006

over the course of weeks or months, whereas TT has a much longer-term onset and is influenced by an accumulation of a lifetime of microbiological and immunological stimuli. Field grading has not previously been standardised across studies and must therefore be compared between studies with caution. TF is used in part for ease and uniformity of field grading rather than specificity for chlamydial infection, and Fig 6 shows that there is a moderate correlation between population *Ct* infection prevalence and population TF prevalence in most districts studied to date. The search criteria for this literature search were relatively lenient; controlling for field grading discrepancy, sample collection methodology, and diagnostic test would likely result in a stronger correlation.

At the individual level, TF and *Ct* prevalence do not always correlate well; for example in a rural Tanzanian community where the TF prevalence was >10%, only 6.1% of those with TF had *Ct* infection and no association was found with clinical signs disease [74]. Follicular inflammation of the conjunctiva can have many different causes, such as viruses, nonchlamydial bacteria, chemical exposure and allergic reactions [76]. While detailed eyelid examination may be able to distinguish these infections phenotypically, the WHO simplified grading system is not sufficiently detailed to do so. *Streptococcus pneumoniae* and *Haemophilus influenzae* have been shown to be significantly associated with TF in the Gambia and Tanzania [74,77]. Numerous other species of the *Staphylococcus*, *Streptococus*, *Moraxella*, *Hameophilus* and *Corynebacteria* genera among others have been cultured from the conjunctivae of children and adults living in trachoma-endemic areas. Although many have not been shown to associate with clinical signs of TF, there are indications that in adults these bacteria can drive

inflammation which leads to increased scar tissue deposition [78] or recurrent TT after surgery [79,80]. It is therefore likely that a proportion of all TF cases globally may not be chlamydial in origin; it seems that in this Pacific Island setting, this proportion is high and this is translated into a reduced prevalence of end-stage trachomatous disease. Fig 4 also indicates that this may also be true of other parts of the world, where districts with sufficient TF to qualify for intervention under WHO guidelines do not necessarily have a significant TT burden. Exposure to circulating *Ct* may modulate the immune response at the conjunctiva to increase inflammation in response to otherwise commensal organisms. In turn this could drive the immunopathology that leads to scarring. It is possible that the low prevalence of *Ct* observed in this population is insufficient to drive intense transmission, and children are exposed less frequently than in other trachoma-endemic populations and therefore are not as susceptible to such intense or regular periods of inflammation.

The highest age-specific TF prevalence in this study was in those aged 6 years (Fig 1). The difference between that and other published data, as documented in Fig 5, may imply a different mode or intensity of transmission, or may reflect reduced accumulation of partial immunity. It is not possible to determine the true mechanism without intensive longitudinal study, but this observation supports the case that the epidemiology of TF in our population is atypical.

While the majority of our *Ct*-positive swabs were taken from eyes with active trachoma, we consider the low absolute number of infections insufficient to drive the moderate burden of TF. It is not clear from our cross-sectional study whether a non-chlamydial microbial agent is causing TF, or whether those with TF had suffered a relatively recent *Ct* infection and had persistent inflammatory disease causing the follicular inflammation we observed. The use of ddPCR has not yet become widespread in infectious disease studies. While it is not suitable for all applications, it offers the significant benefit of reference-free quantification of nucleic acids. In the present study, the load of *Ct* in positive samples was substantial, which are thought to be more transmissible than low-load infection. There was also substantially higher infection load in those with TF as compared to those without TF, although this difference was not statistically significant.

Despite recent advances in culture-free sequence methodologies, low-load infections are known to yield poor quality or no sequence data. The technique we used in this study reportedly provides high quality sequence data (20× read depth over at least 95% of the genome) when the input specimen has above ~12,000 and ~98,000 chlamydial genome copies in vaginal swab and urine samples, respectively [25]. A lower load limit for ensuring high quality sequencing in ocular samples is not yet known, but we were encouraged that partial or complete sequence data were yielded from 11/13 *Ct* positive swabs strains that were sequenced. Those where complete genome coverage (>95%) was achieved appeared to be most closely related to ocular serovars, and appeared to be very closely related to each other. The sequence information suggested that, at *trpA* and the wider PZ, the Solomon sequences were closely related to ocular strains, and ocular and urogenital strains were distinct from each other at these loci. The *trpA* open reading frame was truncated in these sequences. This region contains key determinants of *Ct* tissue tropism and further supports the close relationship of these strains to classical ocular references. The small number of sequences available makes it difficult to identify differences potentially related to pathogenicity. Urogenital strains are known to be able to infect conjunctival epithelium [81], and given the high prevalence of sexually transmitted *Ct* infections in the Solomon Islands [82], we may have expected some contamination of the conjunctivae with urogenital chlamydial strains. Our data did indicate urogenital strains were present in several conjunctivae, but the quality of those sequences aligning to urogenital references was uniformly low. It is not possible to determine whether this was because these

were urogenital strains that had not established a sufficiently fulminant infection to yield enough material for sequencing, or whether the matches obtained were an artefact of the low sequence coverage. We can say, though, that our next generation sequencing confirmed that strains with high sequence homology to well-defined ocular *Ct* strains are present in the conjunctivae of children in the Solomon Islands.

One limitation of our study is the absence of an alternative explanation for the discrepancy between *Ct* and TF levels. Only samples for which testing passed various quality control steps were included in this paper and our test had been previously validated against an external standard. We therefore do not believe that simple diagnostic failure has significantly influenced our data. Of the four signs of trachoma described in this paper (TF, TI, TT and current infection), three (infection, TI and TT) are present at low levels in this population. Further studies are underway to test for potential alternative pathogens such as *S. pneumoniae* and *H. influenzae*, and we are investigating longer-term markers of *Ct* infection by screening the population of Temotu, Rennell and Bellona for both trachomatous scarring and antibodies against chlamydial pgp3 antigens. We have not addressed the genetics of the population in this study and while host genetic factors have been shown to associate with an increased risk of scarring [83,84] very little is known about diversity in immune response genes in the Solomon Islands. The limited amounts of immunogenetic typing data that are available indicate that some HLA epitopes associated with increased risk of scarring (e.g. HLA-C2) are moderately prevalent in the Pacific region, while other putative protective alleles (e.g. the HLA-B*08:01~C*03:04 haplotype) are almost absent (data taken from allelefrequencies.net, search March 2016). Investigating this is beyond the scope of this study, but accumulated evidence on the genetics of trachoma indicate that both pathogen and host are sufficiently well adapted to coexistence that a host polymorphism that makes the host entirely refractory, or pathogen variation that completely ameliorates the infectivity and/or pathogenicity of *Ct* seems unlikely. Polymorphisms in key immune genes such as IL-10 and gamma-interferon have been shown to be more frequent in cases with severe trachoma than in normal controls [85], although these were not replicated in genome-wide association screening. It is possible that variation in immune responsiveness may influence the susceptibility of this population to *Ct* infection but a specific immune pathway that is expressed significantly differently between those in whom scarring progresses and those in whom it doesn't has yet to be identified [75].

In addition to ocular *Ct* infection, we observed signs of both active trachoma and trachomatous conjunctival scarring in this sample indicating trachoma is or has recently been endemic in these islands. However, the prevalence of *Ct* infection appeared to be too low to be the sole explanation for the high burden of TF, while TI and TT were curiously scarce given the substantial amount of TF that was present. Whilst *Ct* may account for some of the TF in this population, we expect that the majority of TF-like disease is either caused by a single as-yet undetermined factor, or by multiple contributory aetiologies. This form of disease, if not unique to the Solomon Islands, might inflate estimates of trachoma burden and could lead to unwarranted mass drug administration in other world populations. In several European settings, there has been a steady increase in incidence and reinfection rates of urogenital *Ct* despite enhanced detection and treatment, which some have hypothesised could be attributed to interruption of the natural acquisition of immunity [86]. We do not have good markers of what constitutes 'acquired immunity to *Ct*' to measure this, but it is relevant in this context to consider the possibility of negative effects of MDA in addition to the potential positive ones. The findings of this study may have profound impacts on approaches to trachoma programme monitoring in the peri-elimination period.

Supporting Information

S1 Checklist. This manuscript adheres to the "Strengthening the Reporting of Observational Studies in Epidemiology (STROBE)" guidelines [89].
(DOC)

S1 Fig. Maximum likelihood phylogram of (**A**) genome and (**B**) plasmid sequences from clinical specimens assembled using *C. trachomatis* E/Bour reference. All branches had bootstrap values over 85/100.
(TIFF)

S2 Fig. Phylogram illustrating relationship of Solomon Islands sequences to reference sequences at (**A**) ompA, (**B**) trpA and (**C**) PZ regions.
(TIFF)

S1 Table. Raw data from this study in accordance with PLoS NTDs editorial guidelines.
(TXT)

S2 Table. Sequence accession numbers.
(DOCX)

Acknowledgments

We are grateful to the survey participants in Rennell, Bellona and Temotu; Elliot Puiahi (National Referral Hospital, Honiara) and Andrew Velaio (Lata Hospital, Lata) for logistical support in the field; Eleanor Challenger and Eleanor Martins (London School of Hygiene & Tropical Medicine, London) for administrative support; and Eleanor Winstanley (Princess Royal University Hospital, Bromley) for logistical support in the UK.

Author Contributions

Conceived and designed the experiments: RMRB OS RTLM DCWM AWS HJT RJW JB ChR.

Performed the experiments: RMRB OS KJ EK LS CR HJT RJW.

Analyzed the data: RMRB CKM MEM RW ChR.

Contributed reagents/materials/analysis tools: RB HT RJW JB ChR.

Wrote the paper: RMRB ChR.

Conducted fieldwork: RMRB OS MM KJ EK LS. Reviewed the manuscript: RMRB OS KJ CKM MEM EK LS CR HJT RJW JB RW RTLM DCWM AWS ChR.

References

1. World Health Organization. WHO Alliance for the Global Elimination of Blinding Trachoma by the year 2020: Progress report on elimination of trachoma, 2013. Wkly Epidemiol Rec. 2014; 96: 421–428.
2. Thylefors B, Dawson CR, Jones BR, West SK, Taylor HR. A simple system for the assessment of trachoma and its complications. Bull World Health Organ. 1987; 65: 477–83. Available: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2491032&tool=pmcentrez&rendertype=abstract PMID: 3500800
3. West SK, Muñoz B, Mkocha H, Hsieh YH, Lynch MC. Progression of active trachoma to scarring in a cohort of Tanzanian children. Ophthalmic Epidemiol. 2001; 8: 137–44. Available: http://www.ncbi.nlm.nih.gov/pubmed/11471083 PMID: 11471083
4. Solomon AW, Peeling RW, Foster A, Mabey DCW. Diagnosis and assessment of trachoma. Clin Microbiol Rev. 2004; 17: 982–1011. doi: 10.1128/CMR.17.4.982–1011.2004 PMID: 15489358

5. Ward B. The prevalence of active trachoma in Fiji. Am J Ophthalmol. 1965; 59: 458–63. Available: http://www.ncbi.nlm.nih.gov/pubmed/14265582 PMID: 14265582
6. Stuppel R. Trachoma in Fiji—an original investigation. Br J Ophthalmol. 1933; 17: 88–97. Available: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=511514&tool=pmcentrez&rendertype=abstract PMID: 18169098
7. Thylefors B. Report on a Field Visit to Kiribati 8–14 April, 1989. Programme for the Prevention of Blindness. Geneva; 1989.
8. Maccallan AF. Trachoma in the British Colonial Empire—its relation to blindness, the existing means of relief, means of prophylaxis. Br J Ophthalmol. 1934; 18: 625–45. Available: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=511737&tool=pmcentrez&rendertype=abstract PMID: 18169234
9. Swanston C. [Trachoma in the Fiji Islands]. Rev Int Trach. 1953; 30: 374–94. Available: http://www.ncbi.nlm.nih.gov/pubmed/13135064 PMID: 13135064
10. Mathew AA, Keeffe JE, Le Mesurier RT, Taylor HR. Trachoma in the Pacific Islands: evidence from Trachoma Rapid Assessment. Br J Ophthalmol. 2009; 93: 866–70. doi: 10.1136/bjo.2008.151720 PMID: 19174394
11. Ngondi J, Reacher M, Matthews F, Brayne C, Emerson P. Trachoma survey methods: a literature review. Bull World Health Organ. 2009; 87: 143–51. Available: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2636192&tool=pmcentrez&rendertype=abstract PMID: 19274367
12. International Association for the Prevention of Blindness. Trachoma Mapping in the Pacific. 2013.
13. Kama M, Cama A, Rawalai K, Koroivueta J. Active Ocular Trachoma In Fiji- A Population Based Prevalence Survey. Fiji J Public Heal. 2013; 2: 11–17.
14. World Health Organization. Report of the 3rd global scientific meeting on trachoma. 19–20 July. Johns Hopkins University, Baltimore, MA; 2010.
15. Last AR, Burr SE, Weiss HA, Harding-Esch EM, Cassama E, Nabicassa M, et al. Risk factors for active trachoma and ocular Chlamydia trachomatis infection in treatment-naïve trachoma-hyperendemic communities of the Bijagós Archipelago, Guinea Bissau. PLoS Negl Trop Dis. 2014; 8: e2900. doi: 10.1371/journal.pntd.0002900 PMID: 24967629
16. Karimurio J, Gichangi M, Ilako DR, Adala HS, Kilima P. Prevalence of trachoma in six districts of Kenya. East Afr Med J. 2006; 83: 63–8. Available: http://www.ncbi.nlm.nih.gov/pubmed/16862999 PMID: 16862999
17. Solomon AW, Pavluck AL, Courtright P, Aboe A, Adamu L, Alemayehu W, et al. The Global Trachoma Mapping Project: Methodology of a 34-Country Population-Based Study. Ophthalmic Epidemiol. 2015; 22: 214–25. doi: 10.3109/09286586.2015.1037401 PMID: 26158580
18. Solomon AW, Holland MJ, Burton MJ, West SK, Alexander NDE, Aguirre A, et al. Strategies for control of trachoma: observational study with quantitative PCR. Lancet. 2003; 362: 198–204. doi: 10.1016/S0140-6736(03)13909-8 PMID: 12885481
19. Melese M, Yi E, Cevallos V, Ray K, Hong KC, Porco TC, et al. Comparison of Annual and Biannual Mass Antibiotic Administration for Elimination of Infectious Trachoma. J Am Med Assoc. 2008; 299: 778–784.
20. House JI, Ayele B, Porco TC, Zhou Z, Hong KC, Gebre T, et al. Assessment of herd protection against trachoma due to repeated mass antibiotic distributions: a cluster-randomised trial. Lancet (London, England). 2009; 373: 1111–8. doi: 10.1016/S0140-6736(09)60323-8
21. Amza A, Kadri B, Nassirou B, Stoller NE, Yu SN, Zhou Z, et al. Community risk factors for ocular Chlamydia infection in Niger: pre-treatment results from a cluster-randomized trachoma trial. PLoS Negl Trop Dis. 2012; 6: e1586. doi: 10.1371/journal.pntd.0001586 PMID: 22545165
22. Derrick T, Roberts C h, Rajasekhar M, Burr SE, Joof H, Makalo P, et al. Conjunctival MicroRNA expression in inflammatory trachomatous scarring. PLoS Negl Trop Dis. 2013; 7: e2117. doi: 10.1371/journal.pntd.0002117 PMID: 23516655
23. Roberts CH, Last A, Molina-Gonzalez S, Cassama E, Butcher R, Nabicassa M, et al. Development and Evaluation of a Next-Generation Digital PCR Diagnostic Assay for Ocular Chlamydia trachomatis Infections. J Clin Microbiol. 2013; 51: 2195–203. doi: 10.1128/JCM.00622-13 PMID: 23637300
24. Last AR, Roberts CH, Cassama E, Nabicassa M, Molina-Gonzalez S, Burr SE, et al. Plasmid copy number and disease severity in naturally occurring ocular Chlamydia trachomatis infection. J Clin Microbiol. 2013; 52: 324. doi: 10.1128/JCM.02618-13 PMID: 24197878
25. Christiansen MT, Brown AC, Kundu S, Tutill HJ, Williams R, Brown JR, et al. Whole-genome enrichment and sequencing of Chlamydia trachomatis directly from clinical samples. BMC Infect Dis. 2014; 14: 591. doi: 10.1186/s12879-014-0591-3 PMID: 25388670
26. Solomon Island Govevrnment. Report on 2009 population and housing census. 2011.

27. R Core Team. R: A Language and Environment for Statistical Computing. In: R Foundation for Statistical Computing [Internet]. 2014. Available: http://www.r-project.org

28. Langmead B, Trapnell C, Pop M, Salzberg SL. Ultrafast and memory-efficient alignment of short DNA sequences to the human genome. Genome Biol. 2009; 10: R25. doi: 10.1186/gb-2009-10-3-r25 PMID: 19261174

29. Li H, Handsaker B, Wysoker A, Fennell T, Ruan J, Homer N, et al. The Sequence Alignment/Map format and SAMtools. Bioinformatics. 2009; 25: 2078–9. doi: 10.1093/bioinformatics/btp352 PMID: 19505943

30. Darling AE, Mau B, Perna NT. progressiveMauve: multiple genome alignment with gene gain, loss and rearrangement. PLoS One. Public Library of Science; 2010; 5: e11147. doi: 10.1371/journal.pone.0011147 PMID: 20593022

31. Cao G, Meng J, Strain E, Stones R, Pettengill J, Zhao S, et al. Phylogenetics and differentiation of Salmonella Newport lineages by whole genome sequencing. PLoS One. 2013; 8: e55687. doi: 10.1371/journal.pone.0055687 PMID: 23409020

32. Schliep KP. phangorn: phylogenetic analysis in R. Bioinformatics. 2011; 27: 592–3. doi: 10.1093/bioinformatics/btq706 PMID: 21169378

33. Paradis E, Claude J, Strimmer K. APE: Analyses of Phylogenetics and Evolution in R language. Bioinformatics. 2004; 20: 289–90. Available: http://www.ncbi.nlm.nih.gov/pubmed/14734327 PMID: 14734327

34. Charif D, Lobry JR. SeqinR 1.0–2: a contributed package to the R project for statistical computing devoted to biological sequences retrieval and analysis. In: Bastolla U, Porto M, Roman HE, Vendruscolo M, editors. Structural approaches to sequence evolution: Molecules, networks, populations. New York: Springer Verlag; 2007. pp. 207–232.

35. Read TD, Brunham RC, Shen C, Gill SR, Heidelberg JF, White O, et al. Genome sequences of Chlamydia trachomatis MoPn and Chlamydia pneumoniae AR39. Nucleic Acids Res. 2000; 28: 1397–406. doi: 10.1086/314538 PMID: 10684935

36. Edgar RC. MUSCLE: Multiple sequence alignment with high accuracy and high throughput. Nucleic Acids Res. 2004; 32: 1792–1797. doi: 10.1093/nar/gkh340 PMID: 15034147

37. Brunelle BW, Sensabaugh GF. The ompA gene in Chlamydia trachomatis differs in phylogeny and rate of evolution from other regions of the genome. Infect Immun. 2006; 74: 578–85. doi: 10.1128/IAI.74.1.578–585.2006 PMID: 16369014

38. Muhammad N, Mohammed A, Isiyaku S, Adamu MD, Gwom A, Rabiu MM. Mapping trachoma in 25 local government areas of Sokoto and Kebbi states, northwestern Nigeria. Br J Ophthalmol. 2014; 98: 432–7. doi: 10.1136/bjophthalmol-2013-303703 PMID: 24344228

39. Jip NF, King JD, Diallo MO, Miri ES, Hamza AT, Ngondi J, et al. Blinding Trachoma in Katsina State, Nigeria: Population-Based Prevalence Survey in Ten Local Government Areas. Ophthalmic Epidemiol. 2008; 15: 294–302. doi: 10.1080/09286580802256542 PMID: 18850465

40. King JD, Jip N, Jugu YS, Othman A, Rodgers AF, Dajom DY, et al. Mapping trachoma in Nasarawa and Plateau States, central Nigeria. Br J Ophthalmol. 2010; 94: 14–9. doi: 10.1136/bjo.2009.165282 PMID: 20385526

41. Mpyet C, Lass BD, Yahaya HB, Solomon AW. Prevalence of and risk factors for trachoma in Kano state, Nigeria. PLoS One. Public Library of Science; 2012; 7: e40421. doi: 10.1371/journal.pone.0040421 PMID: 22792311

42. Mpyet C, Ogoshi C, Goyol M. Prevalence of trachoma in Yobe State, north-eastern Nigeria. Ophthalmic Epidemiol. 15: 303–7. doi: 10.1080/09286580802237633 PMID: 18850466

43. Ramyil A, Wade P, Ogoshi C, Goyol M, Adenuga O, Dami N, et al. Prevalence of Trachoma in Jigawa State, Northwestern Nigeria. Ophthalmic Epidemiol. 2015; 22: 184–9. doi: 10.3109/09286586.2015.1037399 PMID: 26158576

44. Mansur R, Muhammad N, Liman IRN. Prevalence and magnitude of trachoma in a local government area of Sokoto State, north western Nigeria. Niger J Med. 16: 348–53. Available: http://www.ncbi.nlm.nih.gov/pubmed/18080594 PMID: 18080594

45. Cromwell EA, Amza A, Kadri B, Beidou N, King JD, Sankara D, et al. Trachoma prevalence in Niger: results of 31 district-level surveys. Trans R Soc Trop Med Hyg. 2013; 108: 42–8. doi: 10.1093/trstmh/trt101 PMID: 24281748

46. Ngondi J, Onsarigo A, Adamu L, Matende I, Baba S, Reacher M, et al. The epidemiology of trachoma in Eastern Equatoria and Upper Nile States, southern Sudan. Bull World Health Organ. 2005; 83: 904–12. /S0042-96862005001200012 PMID: 16462982

47. Roba AA, Wondimu A, Patel D, Zondervan M. Effects of intervention with the SAFE strategy on trachoma across Ethiopia. J Epidemiol Community Heal. BMJ Publishing Group Ltd; 2010; 65: 626–631. doi: 10.1136/jech.2009.094763

48. Goldschmidt P, Benallaoua D, Amza A, Einterz E, Huguet P, Poisson F, et al. Clinical and Microbiological Assessment of Trachoma in the Kolofata Health District, Far North Region, Cameroon. Trop Med Health. 2012; 40: 7–14. doi: 10.2149/tmh.2011-26 PMID: 22949801

49. Noa Noatina B, Kagmeni G, Mengouo MN, Moungui HC, Tarini A, Zhang Y, et al. Prevalence of trachoma in the Far North region of Cameroon: results of a survey in 27 Health Districts. PLoS Negl Trop Dis. 2013; 7: e2240. doi: 10.1371/journal.pntd.0002240 PMID: 23717703

50. King JD, Odermatt P, Utzinger JJ, Ngondi J, Bamani S, Kamissoko Y, et al. Trachoma among children in community surveys from four African countries and implications of using school surveys for evaluating prevalence. Int Health. 2013; 5: 280–287. doi: 10.1093/inthealth/iht027 PMID: 24179180

51. Schémann JF, Sacko D, Banou A, Bamani S, Boré B, Coulibaly S, et al. [Cartography of trachoma in Mali: results of a national survey]. Bull World Health Organ. 1998; 76: 599–606. Available: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2312488&tool=pmcentrez&rendertype=abstract PMID: 10191556

52. Cruz AA V, Medina NH, Ibrahim MM, Souza RM, Gomes UA, Goncalves GFOR. Prevalence of trachoma in a population of the upper Rio Negro basin and risk factors for active disease. Ophthalmic Epidemiol. 2008; 15: 272–8. doi: 10.1080/09286580802080090 PMID: 18780261

53. Harding-Esch EM, Sillah A, Edwards T, Burr SE, Hart JD, Joof H, et al. Mass treatment with azithromycin for trachoma: when is one round enough? Results from the PRET Trial in the Gambia. PLoS Negl Trop Dis. 2013; 7: e2115. doi: 10.1371/journal.pntd.0002115 PMID: 23785525

54. Harding-Esch E, Jofre-Bonet M, Dhanjal JK, Burr S, Edwards T, Holland M, et al. Costs of Testing for Ocular Chlamydia trachomatis Infection Compared to Mass Drug Administration for Trachoma in The Gambia: Application of Results from the PRET Study. PLoS Negl Trop Dis. 2015; 9: e0003670. doi: 10.1371/journal.pntd.0003670 PMID: 25901349

55. Javaloy J, Ferrer C, Vidal MT, Alió JL. Follicular conjunctivitis caused by Chlamydia trachomatis in an infant Saharan population: molecular and clinical diagnosis. Br J Ophthalmol. 2003; 87: 142–6. Available: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=1771488&tool=pmcentrez&rendertype=abstract PMID: 12543737

56. Zambrano AI, Muñoz BE, Mkocha H, West SK. Exposure to an Indoor Cooking Fire and Risk of Trachoma in Children of Kongwa, Tanzania. Bailey RL, editor. PLoS Negl Trop Dis. 2015; 9: e0003774. doi: 10.1371/journal.pntd.0003774 PMID: 26046359

57. Mkocha H, Munoz B, West S. Trachoma and ocular Chlamydia trachomatis rates in children in trachoma-endemic communities enrolled for at least three years in the Tanzania National Trachoma Control Programme. Tanzan J Health Res. 2009; 11: 103–10. Available: http://www.ncbi.nlm.nih.gov/pubmed/20734706 PMID: 20734706

58. Yohannan J, Munoz B, Mkocha H, Gaydos CA, Bailey R, Lietman TA, et al. Can we stop mass drug administration prior to 3 annual rounds in communities with low prevalence of trachoma?: PRET Ziada trial results. JAMA Ophthalmol. 2013; 131: 431–6. doi: 10.1001/jamaophthalmol.2013.2356 PMID: 23392481

59. Keenan JD, Moncada J, Gebre T, Ayele B, Chen MC, Yu SN, et al. Chlamydial infection during trachoma monitoring: are the most difficult-to-reach children more likely to be infected? Trop Med Int Health. 2012; 17: 392–6. doi: 10.1111/j.1365-3156.2011.02919.x PMID: 22122734

60. Burton MJ, Holland MJ, Makalo P, Aryee EAN, Sillah A, Cohuet S, et al. Profound and sustained reduction in Chlamydia trachomatis in The Gambia: a five-year longitudinal study of trachoma endemic communities. Carvalho MS, editor. PLoS Negl Trop Dis. Public Library of Science; 2010; 4: 10. doi: 10.1371/journal.pntd.0000835

61. Bailey RL, Hampton TJ, Hayes LJ, Ward ME, Whittle HC, Mabey DC. Polymerase chain reaction for the detection of ocular chlamydial infection in trachoma-endemic communities. J Infect Dis. 1994; 170: 709–12. Available: http://www.ncbi.nlm.nih.gov/pubmed/8077735 PMID: 8077735

62. Michel C-EC, Roper KG, Divena MA, Lee HH, Taylor HR. Correlation of clinical trachoma and infection in Aboriginal communities. PLoS Negl Trop Dis. 2011; 5: e986. doi: 10.1371/journal.pntd.0000986 PMID: 21423648

63. Amza A, Kadri B, Nassirou B, Yu SN, Stoller NE, Bhosai SJ, et al. The easiest children to reach are most likely to be infected with ocular Chlamydia trachomatis in trachoma endemic areas of Niger. PLoS Negl Trop Dis. 2013; 7: e1983. doi: 10.1371/journal.pntd.0001983 PMID: 23326612

64. Jenson A, Dize L, Mkocha H, Munoz B, Lee J, Gaydos C, et al. Field evaluation of the Cepheid GeneXpert Chlamydia trachomatis assay for detection of infection in a trachoma endemic community in Tanzania. PLoS Negl Trop Dis. 2013; 7: e2265. doi: 10.1371/journal.pntd.0002265 PMID: 23861986

65. Yang JL, Schachter J, Moncada J, Habte D, Zerihun M, House JI, et al. Comparison of an rRNA-based and DNA-based nucleic acid amplification test for the detection of Chlamydia trachomatis in trachoma. Br J Ophthalmol. 2007; 91: 293–5. doi: 10.1136/bjo.2006.099150 PMID: 17050583

66. Haug S, Lakew T, Habtemariam G, Alemayehu W, Cevallos V, Zhou Z, et al. The decline of pneumococcal resistance after cessation of mass antibiotic distributions for trachoma. Clin Infect Dis. 2010; 51: 571–4. doi: 10.1086/655697 PMID: 20649409

67. Gebre T, Ayele B, Zerihun M, Genet A, Stoller NE, Zhou Z, et al. Comparison of annual versus twice-yearly mass azithromycin treatment for hyperendemic trachoma in Ethiopia: a cluster-randomised trial. Lancet (London, England). 2012; 379: 143–51. doi: 10.1016/S0140-6736(11)61515-8

68. See CW, Alemayehu W, Melese M, Zhou Z, Porco TC, Shiboski S, et al. How Reliable Are Tests for Trachoma?—A Latent Class Approach. Investig Opthalmology Vis Sci. Association for Research in Vision and Ophthalmology; 2011; 52: 6133. doi: 10.1167/iovs.11-7419

69. Bird M, Dawson CR, Schachter JS, Miao Y, Shama A, Osman A, et al. Does the diagnosis of trachoma adequately identify ocular chlamydial infection in trachoma-endemic areas? J Infect Dis. 2003; 187: 1669–73. doi: 10.1086/374743 PMID: 12721948

70. Miller K, Schmidt G, Melese M, Alemayehu W, Yi E, Cevallos V, et al. How reliable is the clinical exam in detecting ocular chlamydial infection? Ophthalmic Epidemiol. 2004; 11: 255–262. doi: 10.1080/09286580490514577 PMID: 15370556

71. Sokana O, Marks M, Butcher R, Solomon AW, Macleod C. The prevalence of trachoma in the Solomon Islands. Ophthalmic Epidemiol. 2015;TBC.

72. World Health Organization. Future approaches to Trachoma Control: Report of a Global Scientific Meeting, Geneva, 17–20 June 1996. World Heal Organ. 1997;

73. Wolle MA, Muñoz BE, Mkocha H, West SK. Constant ocular infection with Chlamydia trachomatis predicts risk of scarring in children in Tanzania. Ophthalmology. 2009; 116: 243–7. doi: 10.1016/j.ophtha.2008.09.011 PMID: 19091415

74. Burton MJ, Hu VH, Massae P, Burr SE, Chevallier C, Afwamba IA, et al. What is causing active trachoma? The role of nonchlamydial bacterial pathogens in a low prevalence setting. Invest Ophthalmol Vis Sci. 2011; 52: 6012–7. doi: 10.1167/iovs.11-7326 PMID: 21693601

75. Burton MJ, Rajak SN, Hu VH, Ramadhani A, Habtamu E, Massae P, et al. Pathogenesis of progressive scarring trachoma in ethiopia and Tanzania and its implications for disease control: two cohort studies. PLoS Negl Trop Dis. 2015; 9: e0003763. doi: 10.1371/journal.pntd.0003763 PMID: 25970613

76. THYGESON P. Etiology and differential diagnosis of non-trachomatous follicular conjunctivitis. Bull World Health Organ. 1957; 16: 995–1011. Available: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2538254&tool=pmcentrez&rendertype=abstract PMID: 13472441

77. Burr SE, Hart JD, Edwards T, Baldeh I, Bojang E, Harding-Esch EM, et al. Association between ocular bacterial carriage and follicular trachoma following mass azithromycin distribution in The Gambia. PLoS Negl Trop Dis. 2013; 7: e2347. doi: 10.1371/journal.pntd.0002347 PMID: 23936573

78. Hu VH, Massae P, Weiss HA, Chevallier C, Onyango JJ, Afwamba IA, et al. Bacterial infection in scarring trachoma. Invest Ophthalmol Vis Sci. 2011; 52: 2181–6. doi: 10.1167/iovs.10-5829 PMID: 21178143

79. Burton MJ, Kinteh F, Jallow O, Sillah A, Bah M, Faye M, et al. A randomised controlled trial of azithromycin following surgery for trachomatous trichiasis in the Gambia. Br J Ophthalmol. 2005; 89: 1282–8. doi: 10.1136/bjo.2004.062489 PMID: 16170117

80. Habtamu E, Wondie T, Aweke S, Tadesse Z, Zerihun M, Zewudie Z, et al. Posterior lamellar versus bilamellar tarsal rotation surgery for trachomatous trichiasis in Ethiopia: a randomised controlled trial. Lancet Glob Heal. 2016; doi: 10.1016/S2214-109X(15)00299-5

81. Mohamed-Noriega K, Mohamed-Noriega J, Valdés-Navarro MA, Cuervo-Lozano EE, Fernández-Espinosa MC, Mohamed-Hamsho J. Conjunctival infection with Chlamydia trachomatis in sexual partners of patients with adult inclusion conjunctivitis. Int Ophthalmol. 2015; 35: 179–85. doi: 10.1007/s10792-014-9930-z PMID: 24643518

82. Marks M, Kako H, Butcher R, Lauri B, Puiahi E, Pitakaka R, et al. Prevalence of sexually transmitted infections in female clinic attendees in Honiara, Solomon Islands. BMJ Open. 2015;In Press.

83. Roberts CH, Molina S, Makalo P, Joof H, Harding-Esch EM, Burr SE, et al. Conjunctival Scarring in Trachoma Is Associated with the HLA-C Ligand of KIR and Is Exacerbated by Heterozygosity at KIR2DL2/KIR2DL3. PLoS Negl Trop Dis. 2014; 8: e2744. doi: 10.1371/journal.pntd.0002744 PMID: 24651768

84. Roberts C h, Franklin CS, Makalo P, Joof H, Sarr I, Mahdi OS, et al. Conjunctival fibrosis and the innate barriers to Chlamydia trachomatis intracellular infection: a genome wide association study. Sci Rep. 2015; 5: 17447. doi: 10.1038/srep17447 PMID: 26616738

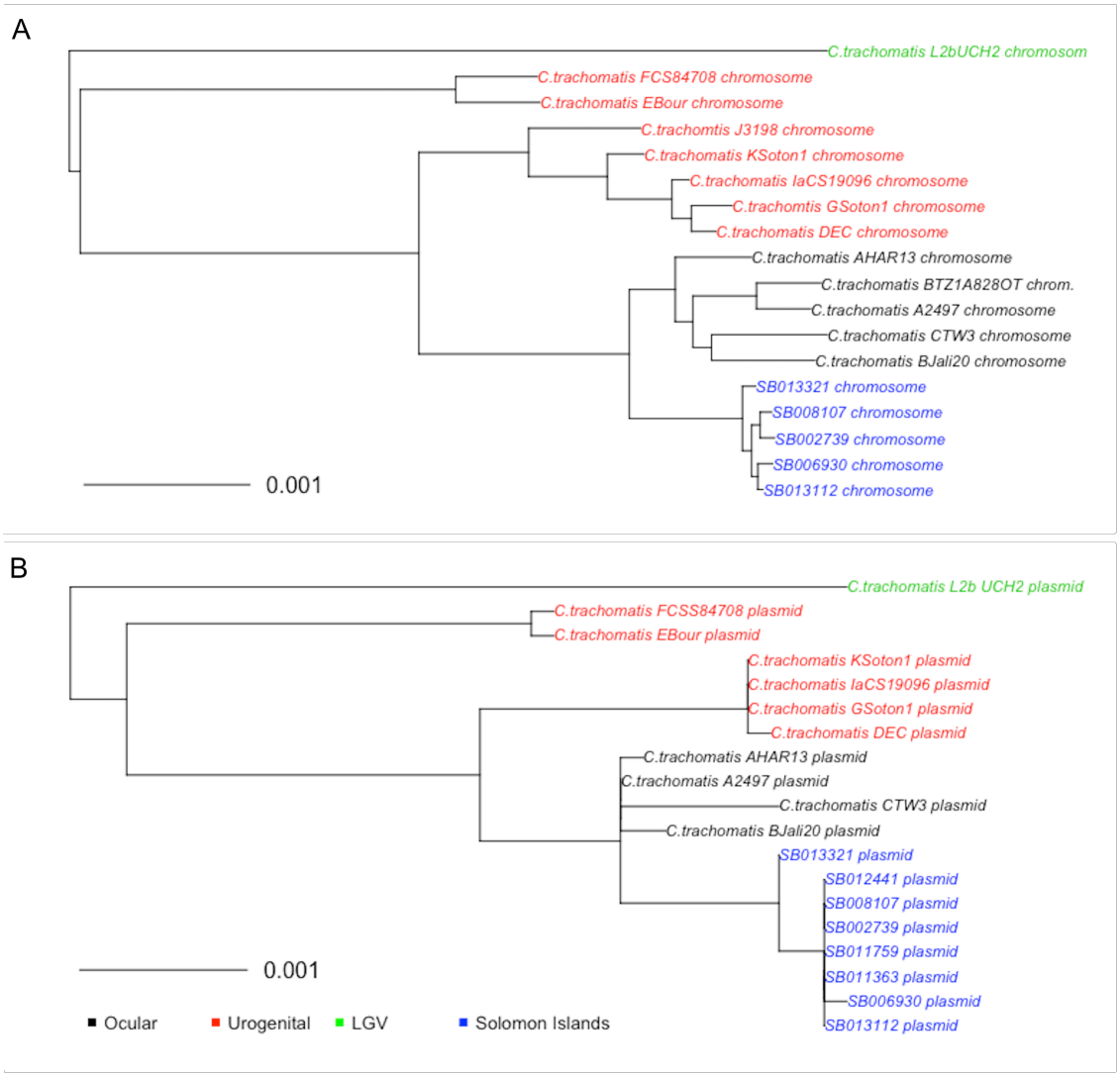
85. Natividad A, Wilson J, Koch O, Holland MJ, Rockett K, Faal N, et al. Risk of trachomatous scarring and trichiasis in Gambians varies with SNP haplotypes at the interferon-gamma and interleukin-10 loci. Genes Immun. 2005; 6: 332–40. doi: 10.1038/sj.gene.6364182 PMID: 15789056

86. Brunham RC, Rekart ML. The Arrested Immunity Hypothesis and the Epidemiology of Chlamydia Control. Sex Transm Dis. 2008; 35: 53–54. doi: 10.1097/OLQ.0b013e31815e41a3 PMID: 18157065

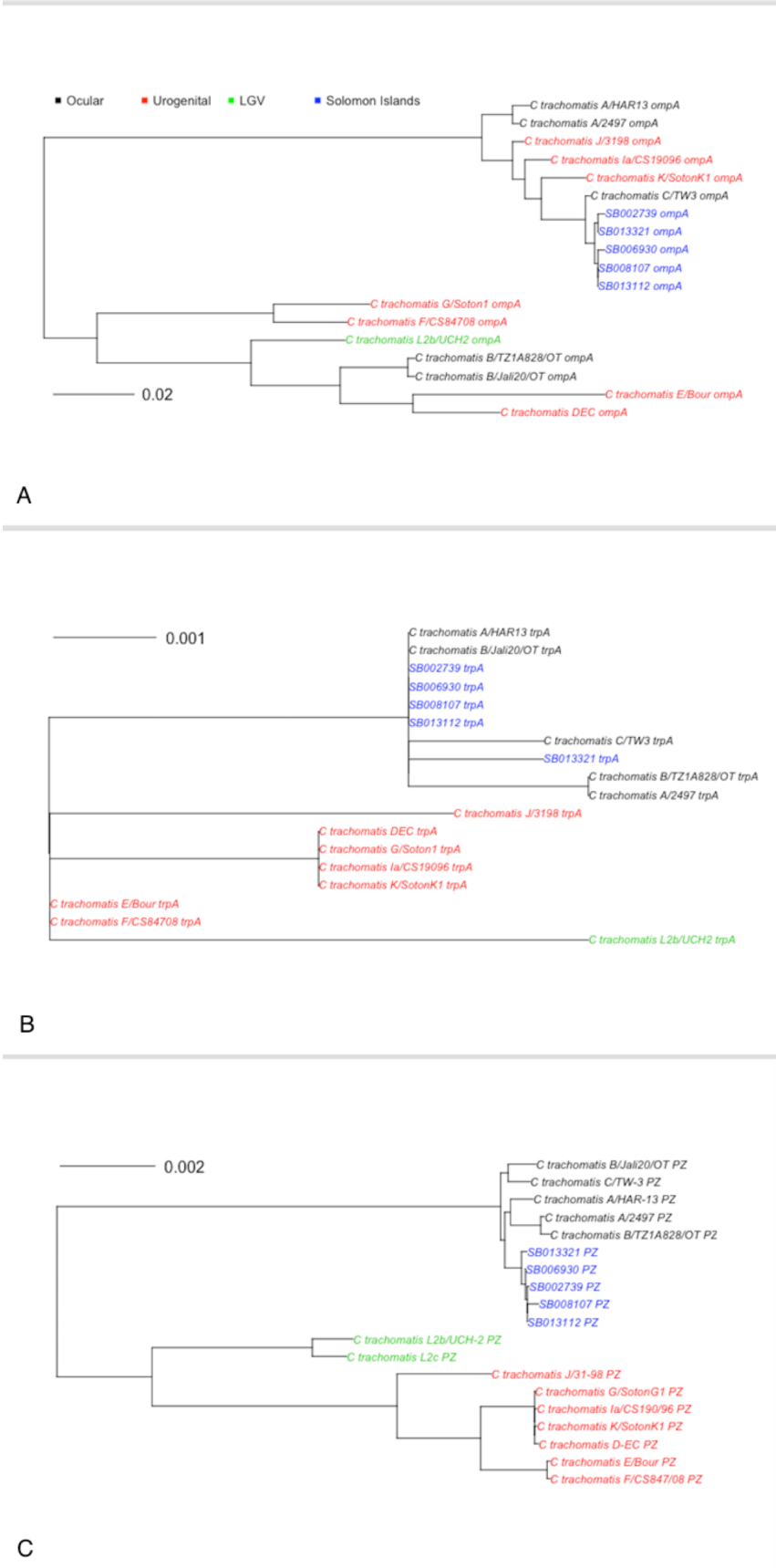
87. Pickett MA, Everson JS, Pead PJ, Clarke IN. The plasmids of Chlamydia trachomatis and Chlamydophila pneumoniae (N16): accurate determination of copy number and the paradoxical effect of plasmid-curing agents. Microbiology. 2005; 151: 893–903. doi: 10.1099/mic.0.27625–0 PMID: 15758234

88. Luo W, Yang H, Rathbun K, Pau C-PC-P, Ou C-YC-Y. Detection of Human Immunodeficiency Virus Type 1 DNA in Dried Blood Spots by a Duplex Real-Time PCR Assay. J Clin Microbiol. 2005; 43: 1851–1857. doi: 10.1128/JCM.43.4.1851–1857.2005 PMID: 15815008

89. von Elm E, Altman DG, Egger M, Pocock SJ, Gøtzsche PC, Vandenbroucke JP. The Strengthening the Reporting of Observational Studies in Epidemiology (STROBE) statement: guidelines for reporting observational studies. Lancet. 2007; 370: 1453–7. doi: 10.1016/S0140-6736(07)61602-X PMID: 18064739



Manuscript supplementary figure 1. Maximum likelihood phylogram of (A) genome and (B) plasmid sequences from clinical specimens assembled using *Chlamydia trachomatis* E/Bour reference.



Manuscript supplementary figure 2. Phylogram illustrating relationship of Solomon Islands sequences to reference sequences at (A) *ompA*, (B) *trpA* and (C) PZ regions.

Manuscript supplementary table 2. Sequence accession numbers.

Participant ID	Chromosome GenBank accession number	Plasmid GenBank accession number
SB002739	CP016418	CP016419
SB006930	CP016420	CP016421
SB008107	CP016422	CP016423
SB013112	CP016424	CP016425
SB013321	CP016426	CP016427

CHAPTER 5: NOTES AND ADDITIONAL INFORMATION

5.3 Note A: Effect of variable storage temperature on swab DNA

5.3.1 Introduction

During the study in section 5.2, swabs were collected into RNAlater (Thermo Fisher Scientific, Carlsbad, USA) and stored for up to 48 hours before freezing. The islands are geographically isolated, have no electricity outside of the provincial capital Lata, and can take several hours of sea travel in outboard-motor canoes on the open ocean to move between islands. It is therefore very difficult to keep specimens frozen. This was achieved by carrying a portable freezer, a generator and 200L barrels of fuel between islands. However, this meant that, for some periods the freezer was closed but not actively being cooled so may have led to some fluctuation in temperature. The internal temperature changed between -60°C and -15°C according to the freezer monitor, therefore never defrosting and justifying the methodology statement in the manuscript. Specimens were transferred to permanent frozen storage at least once every week. However, large variations in temperature, particularly freeze–thaw cycles can damage cells and nucleic acid material, and render them undetectable by NAATs. I set out to ensure that the changes in frozen temperature did not result in specimen degradation sufficient to render them undetectable by PCR. The impact of temperature change on chlamydial material was assessed to determine whether minor cycles of rising and falling frozen temperature caused a significant drop in detected *Ct* load and explain the low *Ct* prevalence.

5.3.2 Methods

5.3.2.1 Swab preparation

Mock positive suspension of *Ct* was prepared as described in section 4.2.3.1. 50μL of 1:500 μL homogenized suspension of serovar A *Ct* EBs and their human host HEp-2 cells was pipetted directly onto 20 sterile swab heads which were immediately placed into RNAlater. The swabs in RNAlater were refrigerated overnight then randomly allocated to one of four storage treatment groups as described in figure 5.3.2.1, five swabs per group. Swabs were variously stored at and cycled between different frozen temperatures (-20°C and -80°C) and defrosting temperature (4°C) depending on the treatment group. The experiment ran for 2 weeks, and then all specimens were allowed to equilibrate to room temperature prior to extraction.

5.3.2.2 Nucleic acid extraction and detection

DNA was extracted using the AllPrep DNA/RNA mini kit, according to manufacturer's instructions. The diagnostic ddPCR assay was carried out as described in the manuscript in section 5.1.

5.3.2.3 Data analysis

Variance and means between groups were compared with one-way analysis of variance (ANOVA).

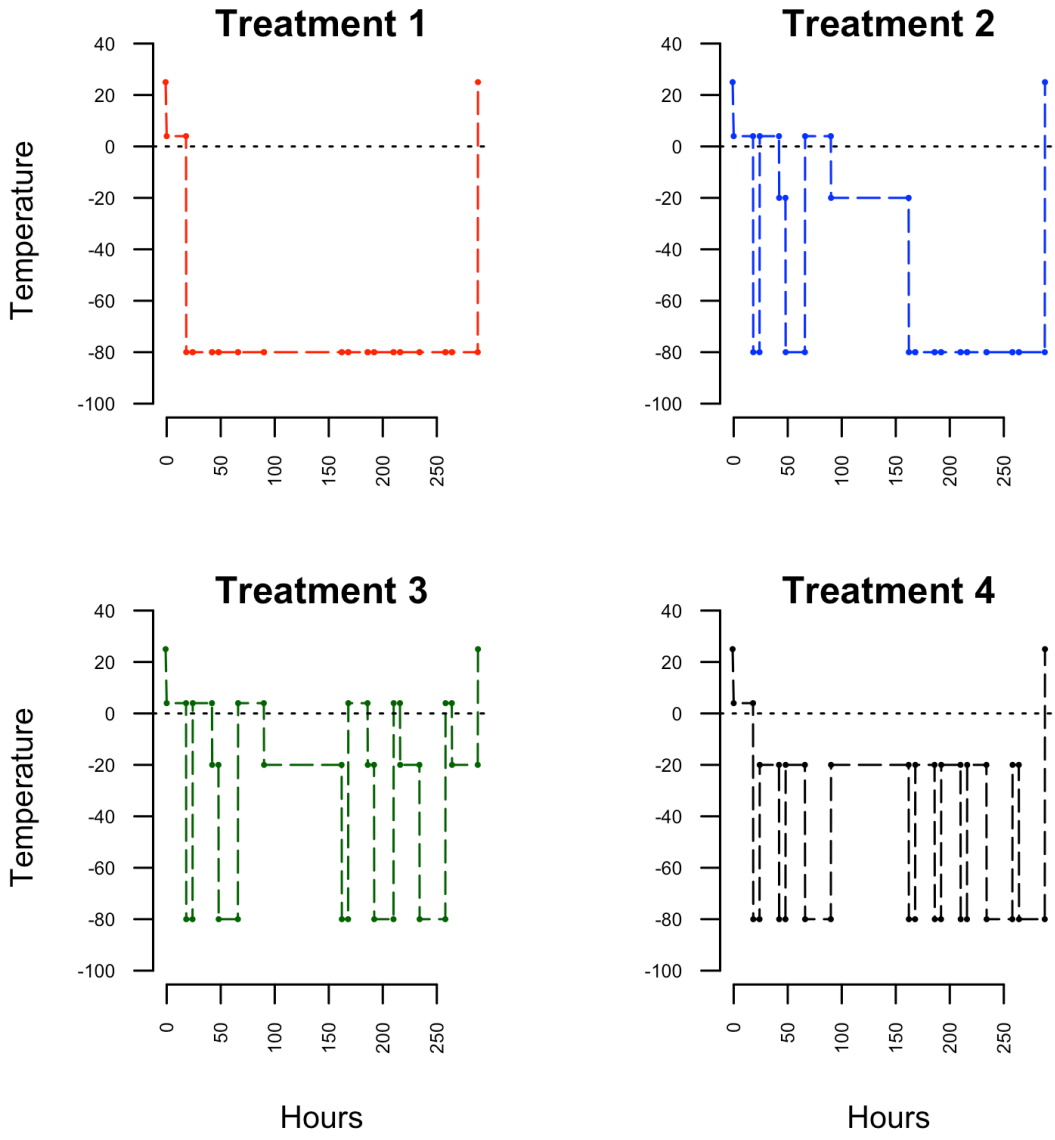


Figure 5.3.2.1. Temperature profiles of treatments for DNA resistance to freeze–thaw study.

5.3.3 Results

Droplet digital PCR failed in one specimen (stored under treatment 4) which was excluded from the analysis. The load detected in swabs stored under the four treatment regimens are displayed in figure 5.3.2.2. There was no significant difference in variance between- and within-groups for either endogenous control load or *Ct* plasmid load (F-statistic = 0.44 and 1.1, respectively) and no significant difference in means overall (p = 0.516 and 0.309, respectively). The null hypothesis for the ANOVA was that all groups have equal variance and means; as no significant differences were found in the overall model, no pairwise t-test was conducted.

5.3.4 Discussion

I am confident that minor fluctuations in the frozen temperature would not lead to diagnostic failure. The low prevalence of detectable *Ct* in this study should not be attributed to changes in storage temperature during specimen collection.

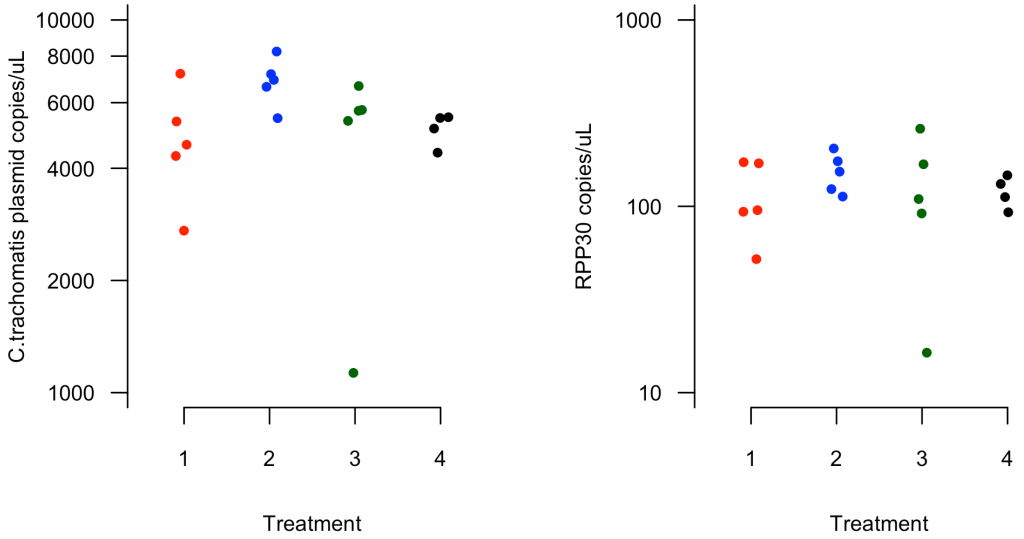


Figure 5.3.2.2. Recovered loads from freeze–thaw study. According to ANOVA testing, none of the differences in the model were significant. Random jitter was added to the x-coordinate of each point to separate points that would otherwise be superimposed.

5.4 Note B: Clustering of clinical trachoma

When calculating the sample size required to assess a given parameter with a specified precision, the sample required from a simple random selection is inflated by a design effect to account for disease clustering (307). The GTMP methodology utilized a general design effect of 2.65 taken from amalgamated previous trachoma surveys (15). From the age profile of TF and the low prevalence of ocular *Ct,* disease transmission and clustering may be expected to differ from that observed in other trachoma-endemic settings. Also, given the geographic isolation of the islands, and even villages within the same island, the spatial distribution of trachoma in this population may also be unique. The inter-cluster correlation coefficient (ICC) was calculated from the between-village, between-household and population-wide variance in TF proportions, where ICC is defined as (307):

$$ICC = \sigma_B / (\sigma_B + \sigma_W)$$

Where σ_B is the between-cluster variance and σ_W is the within-cluster variance. The ICC of TF at the village level was 0.74, and at the household level was 0.55. Both suggest clinical disease is clustered at the household and the village level; more so at the village level. Importantly, this suggests prevalence is unlikely to be homogenous throughout the EU (this is commonly observed in trachoma-endemic areas (232)). The most frequent transmission of infection would be expected at the household level as that would be where most contact took place, however, the higher village-level ICC and the later peak age-specific prevalence could suggest transmission at the community level rather than in the household, such as at school or church.

5.5 Note C: Organism load and genome sequencing success

When the *Ct* genome and plasmid were first sequenced (161), the sequencing technology used required large amounts of input DNA to be able to determine individual bases with confidence. The organisms had to be cultured to generate sufficient genomic material. Culture can take several days per passage and may require several passages from clinical samples. It is time consuming and may lead to some *in vitro* genetic change. As isolates are continuously replicated in the absence of immune pressure and negative selection pressures related to the burden of maintaining such factors, culture can lead to their functional attenuation. Concurrent development of methods for the enrichment of chlamydial DNA without culture and high-throughput, low-cost sequencing (167,168,308) has enabled sequencing of clinical strains directly from diagnostic specimens. In this study, despite the small absolute number of infections, the sequencing success rate from *Ct*-positive samples was relatively high (5/13; 39%). The ddPCR technique provided good estimates of genome copy number in these specimens, this data was therefore used to assess whether a load cutoff for sequencing success could be established in this sample set.

Table 5.5.1 shows a number of indicators of the amount of material collected onto a swab. In the table, more specimens with high *Ct omcB* and plasmid loads appear to have been successfully sequenced than those with low *Ct* loads. The small numbers involved prohibits useful multivariate analysis in this context. This reflects findings from other groups that the absolute number of genome copies, rather than the ratio of *Ct* to other (mostly human, but also other microbial) nucleic acid (167), is the most important factor in determining sequencing success. However, the stated lower limit of reliable successful sequencing for urogenital specimens is between ~12000 and 90000 total genome copies, depending on specimen type (167). In the lowest load specimen to achieve a complete genome sequence, there were an estimated 454 genome copies. This low number might reflect a reduced sample complexity when compared to a urogenital specimen, with fewer microbes and amplification inhibitors than found in the urogenital tract. This should encourage the use of direct sequencing of specimens from ex-diagnostic specimens in trachoma research, as the role of chlamydial genetic polymorphisms in pathogenicity is poorly understood compared to other pathogens (160,166). This is not a reliable limit of detection; reads from two of these specimens with ~24,000 and ~26,000 genome copies, respectively, covered less than 20% of the genome when assembled.

Table 5.5.1. Metrics describing amount of material obtained from swab sample, and their relationship to complete coverage.

Indicator	Median	≥ 95% coverage at 1X depth	<95% coverage at 1X depth
Endogenous control	Above	3	4
	Below	2	3
C. trachomatis plasmid load	Above	4	2
	Below	1	5
C. trachomatis omcB load	Above	4	2
	Below	1	5
DNA concentration	Above	1	5
	Below	4	2

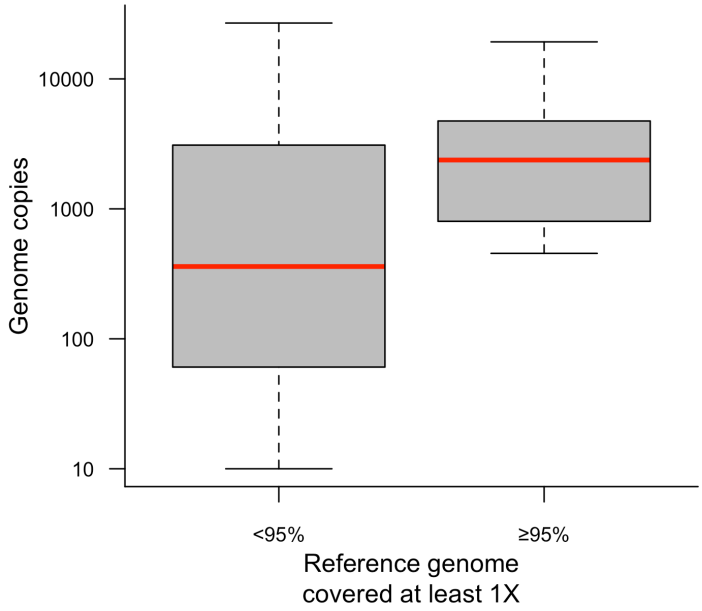


Figure 5.5.1. Genome copies in specimens from which sequence coverage was partial and complete. Genome copies considered equivalent to *omcB* copies. The difference was not significant upon regression testing (p = 0.8). Boxes represent median and inter-quartile range, and the whiskers represent 1.5-times the inter-quartile range.

6. LONG-LIVED SIGNS OF *CHLAMYDIA TRACHOMATIS* TRANSMISSION AND TRACHOMA PATHOGENESIS

6.1 Introduction

In chapter five, the moderate prevalence of the clinical sign TF was not concurrent with highly prevalent infection or TT. This result questions the utility of the WHO simplified grading scheme in the Solomon Islands, and requires further investigation.

The late-stage sequelae of trachoma (i.e. TS, TT) are thought to be more common in people who have had several rounds of recurrent and prolonged *Ct* challenge and inflammation (10). Cross-sectional point estimates of prevalence of transient markers such as TF and *Ct* infection cannot provide insight into historical exposure. In this chapter, two features of trachoma were examined that may reflect historical challenge.

6.2 Anti-Pgp3 ELISA validation: within- and between-assay performance

6.2.1 Introduction

The performance of anti-Pgp3 ELISA is difficult to assess, because true previous-exposure 'positives' and 'negatives' are hard to define. Many *Ct* infections are asymptomatic (227), so it is hard to identify whether a seronegative individual has truly not been exposed to the bacterium. There is also likely to be heterogeneity in production and maintenance of antibody response, so it is difficult to know whether all individuals exposed to *Ct* will become seropositive. Additionally, Pgp3 immunogenicity is not biovar-specific, so there is likely to be an underlying positive rate in any population from urogenital infection and exposure in parturition. TF and infection are not suitable 'gold standards' for traditional diagnostic comparison purposes as they measure transient processes. Additionally, serum level of anti-Pgp3 antibodies is a continuous rather than discrete variable, so the threshold between positive and negative sera is not always clear. The assay was developed at CDC and has been evaluated as part of their work (D Martin *et al*., manuscript under preparation) therefore this section will not focus on the comparability of this data to published datasets, the majority of which have been generated using a multiplex bead array system described elsewhere (243). Prior to rolling the technique out on clinical samples, I set out to determine the intrinsic variability to the ELISA protocol used to increase our confidence in any results generated with the technique.

6.2.2 Methods

6.2.2.1 Dried blood spot collection, preparation and test ELISA

Specimen collection, handling and elution are described in section 6.3. The ELISA protocol used in this study is described in section 6.3.

6.2.2.2 Within-technique reproducibility

The ELISA protocol includes a serial dilution of high anti-Pgp3 titre serum (presumed positive) in low anti-Pgp3 titre serum (presumed negative) on each plate, both sera being supplied by the developing laboratory alongside other reagents. The dilution levels are shown in table 6.2.3.1. Baseline absorbance is removed by running three wells with PBST-milk alone on each plate and subtracting the mean OD from the OD of the other wells of the plate. A successful test was defined as testing on a plate where the OD of standard dilutions fell within 20% of the previously determined mean acceptable values.

This standard dilution series was run in triplicate wells on each of the 20 plates required to run our sample set. The reproducibility of the assay was measured by assessing the variance of these repeated specimens.

All clinical data reported were the first successful result from single extensions from the filter paper wheel eluted in PBST-milk. For 465 specimens randomly selected from the whole specimen set, dried blood was eluted from a second extension and re-tested using the same protocol. The same criteria for eligibility of the test result as described above were applied. The comparison between specimens tested on different plates is enabled by normalization to 200U standard, for which the mean OD value is around 1. Therefore, repeat-tested specimens were normalized prior to comparison.

6.2.2.3 Between-technique reproducibility

A randomly selected subset of 160 specimens were eluted into PBST-milk. An aliquot of PBST-milk was assessed with the protocol developed by the CDC (as described in section 6.3) and then a second aliquot from the same eluate was tested using an independent ELISA technique also assessing Pgp3 reactivity, developed at LSHTM by Harry Pickering.

Pgp3 for the LSHTM ELISA was prepared by culturing *Escherichia coli* transfected with a glutathione S-transferase (GST)-tagged Pgp3-encoding plasmid overnight in 2YT media supplemented with isopropyl β-D-1-thiogalactopyranoside (IPTC) to induce expression. Bacteria were lysed, resuspended in PBS supplemented with 1% Triton X-100 and pelleted to remove cell debris. Soluble GST-tagged proteins were isolated from the supernatant using glutathione sepharose beads, which were then purified by washing from the beads using 5mL polypropylene spin columns. GST tags were removed by incubating with cleavage buffer supplemented with 8% PreScission Protease, and resulting protein eluate purified with chromatography.

A 50-µL aliquot of 1µg/mL Pgp3 in 0.1M sodium bicarbonate (pH 9.6) was added to Immulon 4 HBX microtitre plates (Fisher Scientific, Loughborough, UK) and incubated overnight at 4°C to

bind the antigen. The following day, plates were rinsed twice with washing buffer (PBS with volume/volume 0.05% tween-20, $PBST_{LSHTM}$), and blocked with 100µL blocking buffer ($PBST_{LSHTM}$ supplemented with 2.5% weight/volume nonfat milk power) at room temperature for 4 hours. After two washes, one 50µL aliquot of dried blood spot eluted overnight in PBST-milk was added to each well and incubated at room temperature for 4 hours. After four washes, 100µL anti-human IgG-peroxidase antibody diluted 1/30000 in blocking buffer were added per well and incubated at room temperature for 1 hour. After a final four washes, 100µL 1-Step Ultra TMB-ELISA substrate (Fisher Scientific, Loughborough, UK) was added per well and incubated at room temperature for 10 minutes. The reaction was stopped by addition of 100µL 2M sulphuric acid per well and the OD measured at 450nm for detection and 700nm for background correction.

6.2.2.4 Data analysis

Coefficients of variation (CoVs) were defined as the standard deviation of the mean divided by a mean of repeat-test OD estimates. Intrinsic thresholds for each analysed dataset were predicted using an expectation maximization finite mixture model in the 'mixtools' R package (309,310), whereby a sample was considered positive if the OD was more than three standard deviations above the mean OD of the presumed-negative specimens.

6.2.3 Results

Table 6.2.3.1 shows the variability of repeat testing of ELISA standards. Within plates, the CDC ELISA methodology was highly reproducible across all OD levels, with specimens with ODs above baseline having a mean CoV of 5%. The baseline measurements were more variable with a CoV of 17%. The between-plate variability in standards is a much greater source of variation in assay result.

Table 6.2.3.1. Reproducibility of repeat testing of dilutions of high anti-Pgp3 and low anti-Pgp3 sera.

Standard	High anti-Pgp3 serum	Low anti-Pgp3 serum	Mean* OD	Mean* within-plate CoV	Between-plate CoV
1000U	100%	0%	1.98	5.1%	24.2%
500U	50%	50%	1.50	4.1%	23.9%
200U	20%	80%	1.03	4.5%	24.6%
50U	5%	95%	0.43	6.6%	27.8%
0U	0%	100%	0.13	17.1%	165.6%

CoV: Coefficient of variance; OD: Optical density at 450nm; U: Arbitrary units

*Means determined from mean value across 20 plates of clinical specimens.

Figure 6.2.3.1 depicts the correlation between blood eluted from different blood spots collected consecutively from the same participant. The CoV between different blood spots tested using this assay was 24.8%. The similarity with the between-plate variation estimate suggests the

majority of difference in repeated tested blood spot ODs comes from the assay's inherent inter-plate variation. Upon re-testing, the Spearman's rho correlation coefficient between first and second estimate of Pgp3 level was 0.88, suggesting good agreement. When thresholds were assigned to both datasets independently, 38/465 (8.2%) repeat tested specimens had discrepant results between first and second test, a kappa agreement score of 0.84.

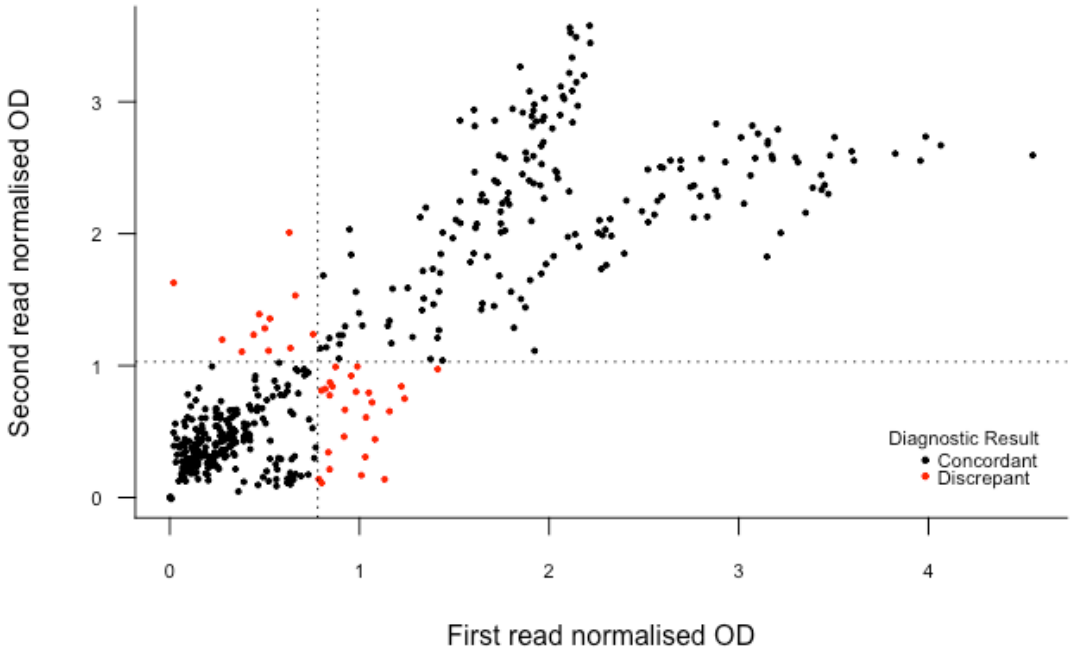


Figure 6.2.3.1. Antibody response to Pgp3 compared between blood spots collected consecutively from the same participant on a single filter wheel. Tested on consecutive days using an identical protocol. Spearman's rho correlation is 0.84, kappa agreement for diagnostic result is 0.84.

Figure 6.2.3.2 demonstrates the correlation between two aliquots of the same blood spot eluate tested using two independent methodologies. The correlation is, in general, good (Spearman's rho 0.88). When thresholds are assigned independently to each dataset, 20/160 (12.5%) specimens tested were discrepant, a Cohen's kappa agreement score of 0.74. Most (19/20) of the discrepant specimens were called positive by the CDC protocol, but negative by the LSHTM ELISA protocol, reflecting higher estimates of OD by the CDC ELISA in the intermediate reactivity range.

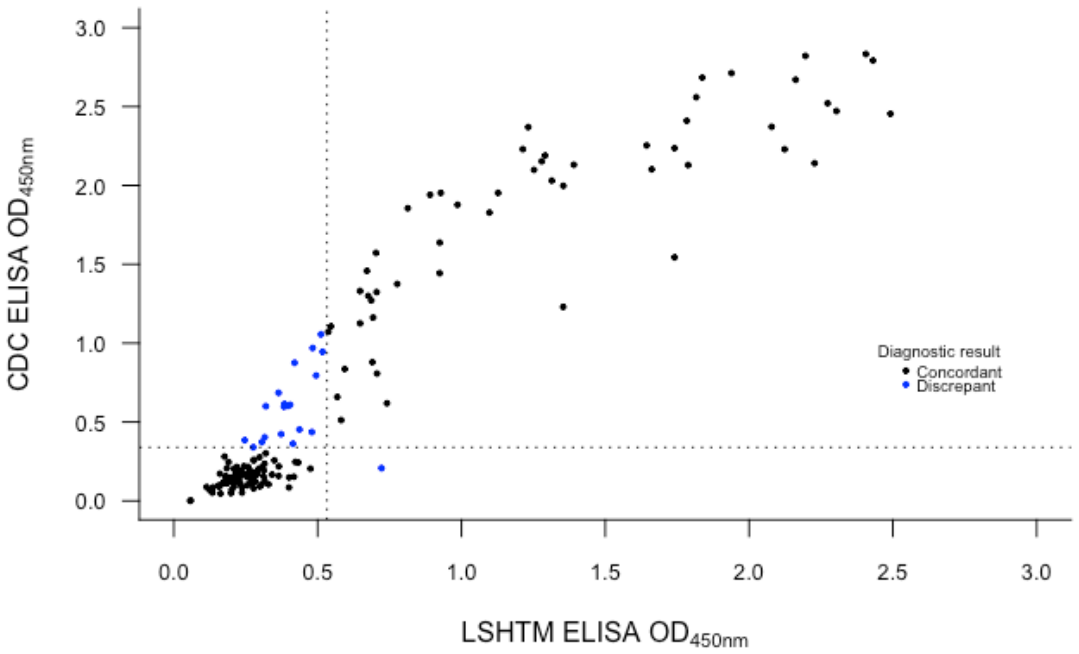


Figure 6.2.3.2. Comparison of eluate optical density estimated by two independent ELISA protocols. Eluate aliquots were tested side-by-side on the same day. Spearman's rho is 0.88, kappa agreement score is 0.74.

6.2.4 Discussion

The estimation of anti-Pgp3 antibody concentration recovered from dried blood spots is inherently variable. The largest contributor to assay variation is between-plate variation, therefore it is appropriate to use a normalization step in the data analysis to ensure the data is comparable to OD data generated by ELISA at other laboratories. Diagnostically, repeat testing samples in this context is likely to create difficulty in defining the result of borderline specimens which intermittently test positive and negative (311). Given the inherent variability of the immune response and the inter-plate variability of the assay, a single well per specimen was considered to be sufficient to estimate reactivity to Pgp3. The comparison with an external ELISA suggests that the estimate of anti-Pgp3 antibodies is generally reliable. However, the CDC protocol generates a slight overestimation of intermediate ODs, leading to a small number of positives that were negative by alternative test. As described above, it is not possible to determine from these data which test is more accurate; it is possible that a number of small protocol differences between the ELISA techniques compared (for example, cleavage of GST tag [LSHTM protocol] compared to nonremoval of the GST tag [CDC protocol] and blocking in PBST-milk [LSHTM protocol] rather than PBST [CDC protocol]) may cumulatively lead to higher absorbance in the CDC protocol. To date, the PPV of IgG anti-Pgp3 antibodies for non-IgG anti-Pgp3 antibodies, or antibodies to other *Ct* antigens has not been assessed. I was satisfied that agreement within- and between-assay was appropriate to estimate the population-level Pgp3 seroprevalence with sufficient accuracy to support the conclusions made in section 6.3.

6.3 Manuscript

In the Solomon Islands, lower age-specific prevalence of anti-Pgp3 antibodies and scarring were found than in comparator regions, and, interestingly, no association was identified between clinical signs of disease and prior *Ct* exposure. A large proportion of TF cases were seronegative and a number of physiologically typical TS cases occurred in seronegative individuals.

We observed relatively little increase in TS cases with age and did not find of evidence of accumulating severe scars with age. Combined with lack of increase in Pgp3 response, this is consistent with a low prevalence of ocular *Ct* infection and low rate of transmission. Both, however, are inconsistent with over 30% of the children in these villages having TF 6 months previously. It is unclear whether the scars observed in Solomon Island children will progress to TT in some individuals. It may be that these scars are caused by acute challenges not related to trachoma and that the immunopathology that leads to TT is not triggered.

Ocular infection with *Ct* is present in the Solomon Islands but the burden appears to be much lower than indicated by the prevalence of TF. Clinical signs of TF and TS are observed in individuals who have no historical exposure to *Ct*. A key unanswered question is what is causing this follicular inflammation if we assume, as the data suggest, that the majority of cases are not related to *Ct*. This will be addressed in chapter seven.

RESEARCH PAPER COVER SHEET

SECTION A – Student Details

Student	*Robert Butcher*
Principal Supervisor	*Chrissy h Roberts*
Thesis Title	*Using alternate indicators to define need for public health intervention for trachoma: Evidence from the Pacific Islands*

If the Research Paper has previously been published, please complete Section B, if not please move to Section C

SECTION B – Paper already published

Where was the work published?	*NA*		
When was the work published?	*NA*		
If the work was published prior to registration for your research degree, give a brief rationale for its inclusion	*NA*		
Have you retained copyright for the work?*	*NA*	**Was the work subject to academic peer review?**	*NA*

If yes, please attach evidence of retention. If no, or if the work is being included in its published format, please attach evidence of permission from the copyright holder (publisher or other author) to include this work.* ***Work published under Creative Commons Attribution 4.0 International Open Access License.*

SECTION C – Prepared for publication, but not yet published

Where is the work intended to be published?	*Lancet Infectious Diseases*
Please list the paper's authors in the intended authorship order:	*Robert M R Butcher, Oliver Sokana, Kelvin Jack, Leslie Sui, Charles Russell, Suzanne Tetepitu, Matthew J Burton, Anthony W Solomon, David CW Mabey, Chrissy h. Roberts.*
Stage of publication	*Under peer review*

SECTION D – Multi-authored work

For multi-authored work, give full details of your role in the research included in the paper and in the preparation of the paper.	*I supported Anthony Solomon in securing funding for this project. I worked with Chrissy Roberts to design this study. I obtained ethical approval for the study. I coordinated the fieldwork with the help of Oliver Sokana, Kelvin Jack, Eric Kalae, Leslie Sui and Suzanne Tetepitu. I conducted the laboratory work independently. I analysed the data with the help of Chrissy Roberts. I prepared the figures and tables, and wrote and revised the manuscript following feedback from co-authors.*

Student signature:  **Date:** 09/12/16

Supervisor signature:  **Date:** 19/12/16

Signs of trachoma are prevalent in Solomon Islanders who have no evidence of prior infection with *Chlamydia trachomatis.*

Robert M R Butcher[1]*, Oliver Sokana[2], Kelvin Jack[2], Diana L Martin[3], Matthew J Burton[1], Anthony W Solomon[1], David CW Mabey[1], Chrissy h. Roberts[1].

[1] Clinical Research Department, London School of Hygiene & Tropical Medicine, Keppel Street, London, WC1E 7HT, UK

[2] Eye Department, Solomon Islands Ministry of Health and Medical Services, P.O. Box 349, Honiara, Solomon Islands

[3] Division of Parasitic Diseases and Malaria, US Centers for Disease Control and Prevention, Atlanta, GA, USA

* Corresponding author: robert.butcher@lshtm.ac.uk, +44 207 927 2419

Keywords

Trachoma; ocular *Chlamydia trachomatis;* ddPCR; anti-Pgp3 antibodies; trachomatous scarring; Solomon Islands

Running header

Chlamydial serology and scarring in the Solomon Islands

Key Messages

- In the Solomon Islands we have identified a disease which has signs that closely resemble those of trachoma (TF, TS) but which occurs in many individuals without serological evidence of prior Ct infection.
- The conjunctival scarring process does not appear to progress to severe scarring and TT, and no consequent threat to vision is evident.
- Clinical grading of TF is shown to be a poor tool to guide MDA in this context. This may have resulted in mass antibiotic distribution here that was not necessary for the purposes of a public health threat from trachoma.
- International guidelines for trachoma elimination should be reassessed in the light of this evidence.

Abstract

Background

The prevalence of trachomatous inflammation-follicular (TF) in the Solomon Islands is moderate, but the prevalence of trachomatous trichiasis (TT) and ocular *Chlamydia trachomatis* (*Ct*) infection is low.

Methods

We enrolled 1511 people aged ≥1 year from randomly selected households in 13 villages in which >10% of the population had TF prior to a single round of azithromycin MDA undertaken six months previously. Presence or absence of signs of trachoma, digital photographs and dried blood spots were collected from each participant. Conjunctival swabs were collected from children aged 1–9 years who had TF. Photographs were analysed for scarring using the modified WHO trachoma grading system. Blood spots were screened for anti-Pgp3 antibodies using enzyme-linked immunosorbent assay. Tests for *Ct* infection were performed on conjunctival swab DNA using droplet digital PCR.

Results

The prevalence of TF in 1–9 year olds decreased to 13.1% from its pre-MDA level of 26.1% ($p<0.0001$). Conjunctival scars were visible in 13.1% of photographs and most severely scarred cases were children. 10% of severe scars had morphology that was atypical for trachoma. Mild ($p<0.0001$) but not severe ($p=0.149$) scars increased in prevalence with age. Anti-Pgp3 antibody prevalence was 18% in 1–9 year olds, sharply increasing around the age of sexual debut, to reach 69% in over 25s. Anti-Pgp3 seropositivity was not associated with scarring in children ($p=0.472$) or TF in children ($p=0.581$). The prevalence of *Ct* infection in TF cases before and after MDA did not differ significantly (3.1% pre-MDA versus 9.8% post-MDA; $p=0.08$).

Conclusions

Signs of trachoma are common in the Solomon Islands but occur frequently in individuals who have no serological evidence of prior ocular infection with *Ct*. WHO recommendations for district-level assessment and MDA provision based on signs alone may have led to inflated estimates of the trachoma burden and mass distribution of antibiotic treatment that was unnecessary for trachoma elimination in this context.

Introduction

Trachoma is responsible for approximately 1.9 million cases of visual impairment or blindness globally.(1) International partners have committed to elimination of trachoma as a public health problem by the year 2020. The causative agent, *Chlamydia trachomatis* (*Ct*), induces specific immune responses at the conjunctiva during an infection(2) which include the polyclonal production of antibodies to *Ct* antigens.(3) Repeated infectious stimuli and the immunological response to them can cause a gradual accumulation of scar tissue in the tarsal conjunctivae.(4) Scarring typically begins to develop in late childhood(5) and can reach a prevalence of 25% in 10-year-olds in hyperendemic populations.(6) Scarring progresses throughout a lifetime and, in severe cases, can lead to entropion, trichiasis, abrasion of the cornea, corneal opacity and blindness.(7)

Global elimination strategies are guided by the signs “trachomatous trichiasis” (TT) and “trachomatous inflammation—follicular” (TF). The World Health Organization (WHO) recommends mass drug administration (MDA) with azithromycin in districts with >5% TF prevalence in 1–9 year-olds.(8) As trachoma control reduces prevalence, the positive predictive value of TF as a *Ct* infection marker drops and phenotypically similar diseases with other aetiologies will be unmasked.(9) We have previously reported data from a 2013 population-based prevalence survey covering the two provinces of Temotu and Rennell & Bellona of the Solomon Islands, which showed the prevalence of TF in those aged 1–9 years was moderately high (26.1%), but TT (0.1%) and ocular infection with *Ct* (1.3%) were rare.(10) In accordance with WHO guidelines, MDA took place in these provinces in 2014.

It was unclear whether the high TF prevalence in these provinces was accompanied by significant burden of trachomatous scarring (TS), whether TF occurred exclusively in those who had previously been infected with *Ct*, or whether MDA would reduce or eliminate TF. Six months after MDA, we revisited villages that previously had high proportions of children with TF and assessed prior exposure to *Ct* infection and age-specific prevalence of scarring.

Methods

Ethics

Study approval was from the London School of Hygiene & Tropical Medicine (LSHTM; 8402) and Solomon Islands National Health Research Ethics Committee (HRC15/03). Subjects aged 18+ years gave written informed consent to participate. A parent/guardian provided consent for those aged under 18 years.

Study design

Thirteen villages in Temotu and Rennell & Bellona provinces were selected because over 10% of the community (all ages) had signs of TF.(10) Due to their small respective populations (Temotu:

21,362; Rennell & Bellona: 3041), the two provinces were combined into one evaluation unit during baseline mapping. The number of villages in each province surveyed as part of this study were selected to reflect the relative population proportion (Temotu: 11 villages; Rennell & Bellona: 2 villages). The survey took place in June-July 2015, six months after a single round of azithromycin MDA had been delivered by the Solomon Islands National Trachoma Elimination Program. The program administered approximately 24000 doses of azithromycin and achieved coverage of approximately 80% in Rennell & Bellona, and 85% in Temotu. After the baseline survey, the National Program also used billboard posters and regular radio spots to promote facial cleanliness and raise awareness of trachoma elimination. The current survey was powered to estimate the prevalence of anti-*Ct* antibody seropositivity in children aged 1–9 years. Based on the low prevalence of ocular *Ct* infection prior to MDA (1.3%), we expected the seroprevalence to be approximately 10%, in line with other communities with low *Ct* prevalence.(11) To estimate seroprevalence with ±5% precision at the 95% confidence level, 367 children were required.(12) In our 2013 survey, we examined a mean of 1.1 children per household and therefore needed 25 households in each of 13 clusters to reach our sample size. All residents aged 1 year or above living in households drawn at random from a list of all households in a study cluster were eligible to participate.

Trachoma grading

Note A - page 140

Grading using the WHO simplified system(13) for TF, ”trachomatous inflammation—intense” (TI) and TT was performed in the field by one of two Global Trachoma Mapping Project (GTMP)-certified graders, wearing 2.5× binocular magnifying loupes.(14) High-resolution digital photographs of the right tarsal conjunctivae were graded for TS using the modified WHO trachoma grading system (Table 1).(15) Photographs were graded by two photo-graders who had previously achieved kappa scores for inter-observer agreement of >0.8 for F, P and C (follicles, papillae and cicatricae) grades, compared to a highly experienced trachoma grader. Photograph grading was undertaken masked to field grading, laboratory results, and the other photograph grader’s assessment. Discrepant grades were arbitrated by a third highly experienced grader.

Specimens

Sterile lancets (BD Life Sciences, Oxford, UK) were used to collect 10 µL of blood onto filter paper (CellLabs, Sydney, Australia) which was air-dried for 4–12 hours before being sealed in ziplock bags with desiccant sachets. These were refrigerated for up to one week and then stored at -20°C before shipping at ambient temperature to LSHTM.

Using standard methods,(16) right conjunctival swabs were collected from any children aged 1–9 years who had signs of TF and/ or TI. Swabs were frozen to -20°C within one week of collection and shipped to LSHTM on dry ice for processing.

Serological and nucleic acid testing

Previous exposure to *Ct* infection was measured by detection of anti-*Ct* Pgp3 antibodies using an enzyme-linked immunosorbent assay (ELISA).(17)(18) Optical density (OD) at 450 nm was measured using SpectraMax M3 photometric plate reader (Molecular Devices, Sunnyvale, USA).

DNA was extracted from swabs with the QIAamp DNA mini kit (Qiagen, Manchester, UK). We tested samples for *Homo sapiens* ribonuclease subunit (RPP30 endogenous control) and open reading frame 2 of the *Ct* plasmid (diagnostic target) using a droplet digital PCR assay(19) with minor modifications.(20)

Data analysis

All data analyses were conducted using R 3.2.3.(21) Pre- and post-MDA proportions were compared using Wilcoxon's rank sum test. ddPCR tests for current ocular *Ct* infection were classified into negative and positive populations according to methods described previously (19). ELISAs for antibodies to *Ct* were classified as negative or positive using an expectation-maximisation fixed mixture model (Migchelsen et al, in press). Using this method, the threshold normalised OD value for positivity was 0.7997.

Results

Study demographics

In total, 1511 people (46.3% male; 466 1–9 year-olds) aged 1 year and over were examined in 382 households from the 13 selected study villages. By comparison, the pre-MDA survey of the same villages yielded 1534 people (490 1–9 year-olds) in 394 households. Data on non-participation were not collected in June 2015, but the number enrolled was similar to that for the pre-MDA survey, suggesting a similar participation rate (~90%) on both occasions. In this study, there was a mean of 4 people per household aged 1 year and over, and a mean of 1.2 children per household aged 1–9 years, which, after accounting for non-participants, are similar to the means in the 2009 Solomon Islands National Census (4.9 people of any age and 1.4 children aged 1–9 years per household in Temotu, 4.4 people of any age and 1.1 people aged 1–9 years per household in Rennell & Bellona).(22)

Active trachoma and TT

Prior to MDA, there were 165/489 (33.7%) cases of TF in either eye and 1/489 (0.2%) case of TI in those aged 1–9 years in study villages.(10) Following MDA, we observed 66/466 (14.2%) cases of TF and no cases of TI in either eye, representing a decrease in TF of 58% ($p<0.0001$). A similar pattern was observed in right eyes considered alone – the eyes from which swabs were collected if indicated (Table 2). 56% of TF cases following MDA were bilateral. No cases of TT were identified during this study.

In the two enrolled villages of Rennell & Bellona, a slight increase in the prevalence of TF in either eye in those aged 1–9 years following MDA was noted, but it was not statistically significant (11/60 [17.9%] before MDA to 14/78 [18.3%] after MDA; $p=0.956$). In contrast, in the 11 enrolled villages of Temotu, a substantial decrease in TF (from 155/430 [36.0%] before MDA to 52/388 [13.4%] after MDA; $p<0.0001$) was observed.

Trachomatous scarring

Note B - page 141

Of the right eye photographs collected, 1440/1511 (95.3%) were suitable for grading. 188/1440 (13.1%) photographs were graded as C>0, of which 127 were C1, 53 were C2 and 8 were C3. Exemplars of scarring that resembled normal trachoma phenotypes are shown in Figures 1A and 1B. The photo-graders noted that some conjunctivae met the criteria for C3, with clear bands of scarring, but also showed clear features not typically associated with trachomatous pathology. In some cases, these were characterised by boundaries demarcating heavily scarred from apparently healthy conjunctiva (Figure 1C and 1D). Photograders noted atypical scars in 4/53 (7.5%) C2 cases and 3/8 (37.5%) C3 cases. Of the scarred eyelids classed as typical for trachoma, 36/54 (67%) were seropositive, whereas 2/7 (29%) of those classed as ‘atypical’ were seropositive, although the difference in proportions was not significant (chi-squared test $p=0.123$).

Figure 1. Photographs from adults graded as C3. (A and B). Conjunctivae with features meeting the criteria for C3 characteristic of trachoma (C and D). Conjunctivae with features meeting the criteria for C3 but thought not to be trachomatous in origin. Study IDs were SB113564, SB108878, SB107613 and SB108669. All taken of residents in 13 selected communities of Temotu and Rennell & Bellona Provinces, Solomon Islands, June-July 2015.

The age-specific prevalence of scarring is shown in Figure 2. Of 435 photographs graded from children aged 1–9 years, 25 (5.7%) were graded as C>0. In 311 adults aged >40 years who were examined, 74 (23.8%) had C>0 (65 cases of C1, 9 cases of C2, 0 cases of C3). Of 8 cases of C3 in the population, 4 (50%) were in children aged 1–9 years, although 2 of these were classed as 'atypical'.

The proportion of people with C1 increased with age (logistic regression $p<0.0001$) but the proportion of people with more severe scarring (C2 or C3) did not increase with age (logistic regression $p=0.149$). There was no significant association between having C>0 and gender (chi-squared test $p=0.80$). In Rennell & Bellona, 25/225 (11.1%) of photos were graded C>0, whereas in Temotu, 163/1215 (13.4%) of photos were graded C>0; the difference in scarring between provinces was not significant (Wilcoxon Rank Sum test $p=0.289$).

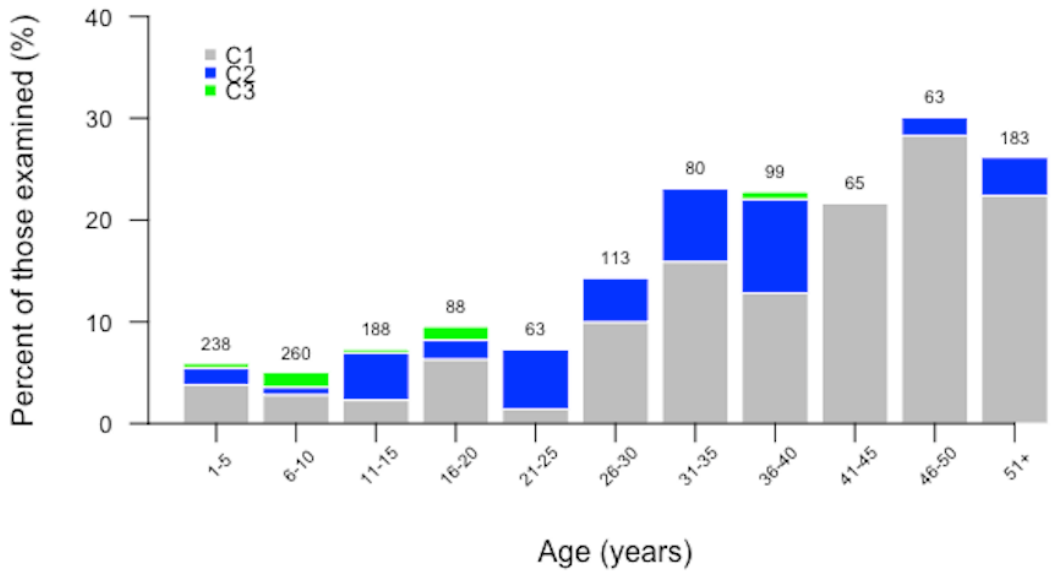


Figure 2. Age-specific prevalence of scarring (defined as C>0), as identified by photographic grading, in 13 selected communities of Temotu and Rennell & Bellona Provinces, Solomon Islands, June-July 2015. Bar labels represent total number of people in each age group.

Anti-Pgp3 serology

Note C - page 144

Dried blood spots were collected from 1499/1511 (99.2%) people of all ages during the post-MDA survey; the other 12 people declined finger-prick. Overall, anti-Pgp3 seroprevalence was 633/1499 (42.2%). In children aged 1–9 years, the prevalence of anti-Pgp3 antibodies was 83/462 (18.0%); in 1-year-olds alone, it was 5/47 (10.6%). The mean seroprevalence in those aged 6–10 years was not significantly higher than in those aged 1–5 years (20.3% compared to 16.6%, Wilcoxon rank sum p=0.276). The largest increase in seroprevalence was observed between those aged 16–20 years and 21–25 years when the seroprevalence rose significantly from 30.4% to 71.6% (Wilcoxon rank sum p<0.0001). Of those aged over 25 years, 67.4% were seropositive (Figure 3). In the 16–20-year-old age group, the prevalence of seropositivity amongst females was higher than in males (13.9% versus 41.1%, Wilcoxon rank sum test p<0.0001). The seroprevalence among children in Rennell & Bellona was significantly higher than that in Temotu (38.5% versus 13.8%; chi-squared p<0.0001).

There was no association between seropositivity and signs of TF in children aged 1–9 years (19.7% seropositive in those with TF in either eye compared to 17.7% seropositive in those without TF, p=0.581). In those younger than the median self-reported age of sexual debut (18 years (23)), there was no association between C grade and anti-Pgp3 OD (linear regression

adjusted for age and gender p=0.453) or anti-Pgp3 positivity (logistic regression adjusted for age and gender p=0.472).

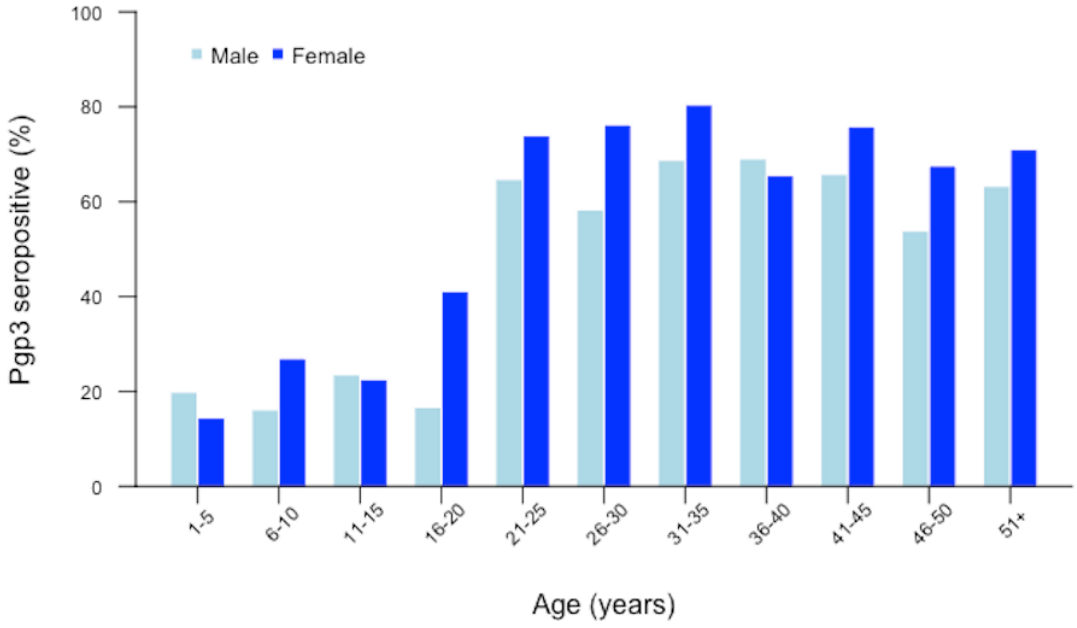


Figure 3: Age-specific seroprevalence of anti-Pgp3 antibodies, separated by gender. Pale blue bars represent proportion of seropositive females in each age group (optical density over 0.7997), dark blue bars represent proportion of seropositive males. Dried blood specimens collected from 13 selected communities of Temotu and Rennell & Bellona Provinces, Solomon Islands, June-July 2015.

Ocular *C. trachomatis* infection

Swabs from all 61 children aged 1–9 years with TF in the right eye that were tested for *Ct* had a positive endogenous control result; the median load of the human RPP30 target was 83,000 copies, equivalent to over 40,000 conjunctival cells. In this study, 6/61 (9.8%) of children with active trachoma had *Ct* infection. Of the 6 specimens from children positive for *Ct,* the median load was 104,100 plasmid copies/swab. We previously showed that before MDA, 5/160 (3.1%) of those with active trachoma in study villages had evidence of infection with *Ct.* The median pre-MDA load of *Ct* infections in these villages was 51,880 plasmid copies/swab.(10) Neither the difference between the pre- and post-MDA *Ct* prevalence nor pre- and post-MDA *Ct* load was statistically significant (Wilcoxon rank sum test p=0.08 and p=0.22, respectively). The relationship between *Ct* infection, signs of trachoma and seropositivity was examined in children aged 1–9 years and is summarised in Table 3. All six cases of active trachoma in which infection was also detected were in seropositive individuals (Figure 4). All study villages had at least one case of TF, but infections were limited to three of the 13 villages studied. Two villages in Rennell & Bellona housed five of the six *Ct* infections identified during this study.

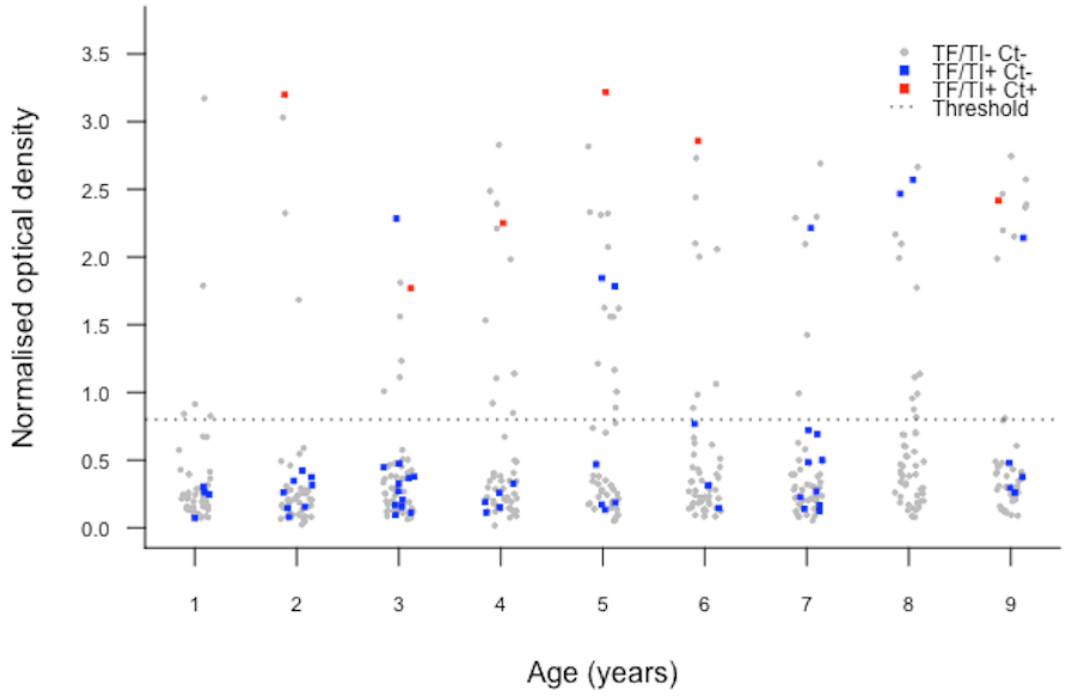


Figure 4: Anti-Pgp3 antibody titre from children aged 1–9 years in 13 selected communities of Temotu and Rennell & Bellona Provinces, Solomon Islands, June-July 2015. Those with trachomatous inflammation—follicular (TF) and/or trachomatous inflammation—intense (TI) but no infection are highlighted in blue, those with TF and/or TI and infection are red and those with neither TF nor TI nor infection are grey. The horizontal dotted line represents the threshold for seropositivity. Random jitter was applied to the x-coordinate of each data point to separate points that would otherwise overlap.

Discussion

The Solomon Islands, along with other Pacific Island states, has been identified as trachoma-endemic based on moderately high province-level prevalences of TF. Whilst measures for trachoma elimination have already been deployed in Temotu and Rennell & Bellona, we have previously noted that TI, ocular *Ct* infection and late-stage disease (TT) are rare.(10) If the findings from these study villages were replicated throughout the district, TF would still be sufficiently prevalent to warrant intervention. However, using a suite of non-TF tools (clinical photography for evaluation of conjunctival scarring, nucleic acid infection testing and serological testing), we have demonstrated here that ocular *Ct* is scarce and is not being widely transmitted, and that TF is not concurrent with prevalent severe scarring or TT in this population.

We would not expect to see large numbers of individuals with TF who have not previously been infected with *Ct*, but in this population, the majority (80.3%) of individuals with TF were seronegative, and participants with TF were no more likely to be seroreactive to Pgp3 than their peers without TF. We found a small and non-significant increase in age-specific seroprevalence between young children (0–5 years) and older children (6–10 years), which suggests that there is limited horizontal transmission of *Ct* strains among children. This is concordant with our previous data, which suggested that although ocular *Ct* strains are present in the Solomon Islands, they are rare.(10) The increase in seropositivity with age in this group was modest compared with that seen in hyperendemic villages of the United Republic of Tanzania, where seropositivity has been observed to increase from approximately 25% to 94% between the ages of 1 and 6 years.(17) In the current dataset, there was a rapid increase in age-specific seroprevalence around the age of 18 years, the self-reported median age of sexual debut in a nearby population.(24) The prevalence of urogenital *Ct* infection is known to be high in women attending antenatal clinics in the Solomon Islands (24), which may account for the high seroprevalence in adults, and exposure during parturition may also be a major contributor to the 10% of 1-year-olds in our study who had evidence of prior Pgp3 exposure.(25)

Antibodies to Pgp3 have recently been suggested for monitoring *Ct* transmission in trachoma programmes,(26,27), but there is still much to learn about the dynamics of these responses. For example, it is not clear whether anti-Pgp3 responses are detectable in all individuals previously infected with *Ct,* or if multiple exposures are required to develop sustained anti-*Ct* responses. In this study, only 19.7% of children with TF were positive for anti-Pgp3 antibodies, but all six children with current infection were seropositive. There was also a high prevalence of Pgp3 reactivity in adults living in Temotu and Rennell & Bellona, a proportion of whom are likely to have had a previous urogenital *Ct* infection.(24) While seroreversion due to clearance of infection by MDA is a possible explanation for the low seroprevalence and absence of association of anti-Pgp3 antibodies with TF, there is currently no evidence for complete seroreversion for Pgp3-specific antibodies(17,28) after clearance of infection. We are therefore confident that Pgp3 is an appropriate antigen for serosurveillance in this population.

Analysis of the age-specific prevalence of conjunctival scarring illustrated that, while the proportion of people with mild scars increased with age, the proportion of those with more extensive or eyelid-distorting scars did not increase with age. Contrary to what might be expected in a trachoma-endemic community,(29) no eyelid-distorting scars were found in 311 adults >40 years. There does appear to be severe scarring among children, but some cases are atypical (Figure 1) and are in children who lack Pgp3 reactivity (Table 3), so other causes of scarring may be contributing to this (presumed) ongoing incidence. There are a number of inflammatory conditions (e.g. adenoviral, acute haemorrhagic or membranous conjunctivitis) which may result in conjunctival scarring, although the pathology, incidence and prevalence of these are poorly understood.(30) It is currently unclear whether the TF that we observed is directly linked to conjunctival scarring in this setting. Currently in Temotu and Rennell & Bellona, the low prevalence of severe scars suggests that the proportion of the population at risk of developing TT is very low, although we cannot determine how this might change temporally.

Prior to intervention with MDA, more than 15% of children living in the selected villages had TF. The 13 communities included here were the most highly endemic of those surveyed in Temotu and Rennell & Bellona during the GTMP. In this study, we showed that the burden of TF in many of these villages dropped significantly following a single round of MDA, but still remains above the threshold for continued intervention. The drop in clinical disease was not reflected by a drop in ocular *Ct* in children with TF, which increased, albeit statistically insignificantly. From interventions in other settings, we might expect TF prevalence to drop by approximately 50% six months after a single round of MDA, given 80% population coverage.(31,32) Azithromycin has anti-inflammatory and broad-spectrum antibiotic effects, which may help explain the observed decrease in clinical disease. We observed regional variation across the study villages. Compared to Temotu, we noted more children were seropositive, more children with TF had infection, and MDA did not have as significant an impact on TF levels in Rennell & Bellona. Our survey was not prospectively designed to assess these differences, and the subgroup size in Rennell & Bellona precludes more detailed analysis. Temotu is much more similar to the rest of the Solomon Islands in terms of the geology of the islands, and the lifestyle and ethnicity of the majority of the inhabitants. Further studies on the localisation of trachoma in the islands are warranted.

The complex, multistage nature of trachoma makes it difficult to predict the outcome of a given intervention.(33) Data from cross-sectional surveillance tools used in isolation can be hard to interpret, especially given the prolonged persistence of TF after clearance of infection.(34) Some features of conjunctivitis in the Solomon Islands resemble trachoma, particularly the prevalent follicular inflammation and some of the severe conjunctival scarring. Crucially, these clinical features were not co-endemic with TT at a prevalence that indicates a public health problem. In this setting, tests for infection gave a better indication of the public health threat from trachoma than TF. A combined approach in which various age-specific markers of trachoma are assessed together across the complete age range of the population, may prove useful for prioritising areas for intervention where the prevalence of TF alone does not coherently reflect trachoma's public health importance.

Our data provide compelling evidence that trachoma is not a public health problem in these villages. Whilst there have been substantial collateral benefits to local residents from having received MDA (such as on genital *Ct*(35) and yaws(36)), further rounds of azithromycin MDA do not appear to be indicated for the purposes of trachoma elimination. WHO recommendations for implementation of MDA and the SAFE strategy should be reviewed in the light of this evidence.

Acknowledgements

We are grateful to the survey participants in Temotu and Rennell & Bellona, to Andrew Velaio for logistical support in the field, and to Leslie Sui, Charles Russell and Suzanne Tetepitu for helping to conduct the field work. The findings and conclusions in this report are those of the authors and do not necessarily represent the official position of the Centers for Disease Control and Prevention.

Financial statement

The field and laboratory costs were funded by the Fred Hollows Foundation, Australia (1041).

RMRB and AWS were funded by a Wellcome Trust Intermediate Clinical Fellowship to AWS (098521).

OS and KJ were employed by the Solomon Islands Ministry of Health and Medical Services for the duration of the survey.

DLM receives funding through the US Agency for International Development through an interagency agreement with CDC.

MJB was funded by the Wellcome Trust (098481/Z/12/Z).

ChR is supported by the Wellcome Trust Institutional Strategic Support Fund (105609/Z/14/Z).

The funders had no role in the design, execution or publication of this study.

Author Contributions

Conceived and designed the study: RMRB, OS, DLM, AWS, DCWM, ChR.

Performed the fieldwork: RMRB, OS, KJ

Provided training and reagents: DLM

Performed the experiments: RMRB, MJB, ChR

Analysed the data: RMRB, MJB, ChR

Wrote the manuscript: RMRB, ChR

Revised and approved the manuscript: RMRB, OS, KJ, DLM, MJB, AWS, DCWM, ChR

Tables

Table 1. Grading system for conjunctival scarring, reproduced from Dawson and colleagues (15).

Grade	Classification	Definition
C0	Absent	No scarring on the conjunctiva
C1	Mild	Fine scattered scars on the upper tarsal conjunctiva, or scars on other parts of the conjunctiva
C2	Moderate	More severe scarring but without shortening or distortion of the upper tarsus
C3	Severe	Scarring with distortion of the upper tarsus

Table 2. Population characteristics of study populations before and after MDA, 13 selected communities of Temotu and Rennell & Bellona Provinces, Solomon Islands.

Characteristic	**Pre-MDA (October–November 2013;** (10)**)**	**Post-MDA (June–July 2015: this study)**	**p-value***
Number examined, all ages	1534	1511	-
Number examined aged 1–9 years	490	466	-
Number of households enrolled	394	382	-
% male of those examined	46.5	46.3	0.836
TF in either eye in 1–9 year olds	33.7%	14.2%	<0.0001
Active trachoma in swabbed eye (right eye field assessment)	160 (32.7%)	61 (13.1%)	<0.0001
Ct infection in those aged 1–9 years	5 (3.1%)	6 (9.8%)	0.08
Median *Ct* infection load in positive specimens (plasmid copies/swab)	51,880	104,100	0.219

Ct: Chlamydia trachomatis

* t-test

Table 3. Serological status compared to other tests for trachoma, 13 selected communities of Temotu and Rennell & Bellona Provinces, Solomon Islands, June–July 2015

Comparator		1–9 year-olds			≥10 year-olds		
		Seronegative	**Seropositive**	**Total**	**Seronegative**	**Seropositive**	**Total**
Ct infection by ddPCR*	Positive	0	6	6	-	-	-
	Negative	48	7	55	-	-	-
TF	Positive	53	13	66	13	9	22
	Negative	326	70	396	474	541	1015
TI	Positive	0	0	0	0	1	1
	Negative	379	83	462	487	549	1036
Scarring	C0	333	77	410	414	418	832
	C1	15	1	16	36	75	111
	C2	3	2	5	16	32	48
	C3	3	1	4	1	3	4

Ct: Chlamydia trachomatis; ddPCR: droplet digital polymerase chain reaction; TF: trachomatous inflammation—follicular; TI: trachomatous inflammation—intense.

* presence or absence of infection only assessed in children with TF and/or TI.

1. Bourne RA, Stevens GA, White RA, Smith JL, Flaxman SR, Price H, et al. Causes of vision loss worldwide, 1990-2010: a systematic analysis. Lancet Glob Heal. 2013 Dec;1(6):e339-49.

2. Burton MJ, Rajak SN, Bauer J, Weiss HA, Tolbert SB, Shoo A, et al. Conjunctival transcriptome in scarring trachoma. Infect Immun. 2011 Jan;79(1):499–511.

3. Kari L, Bakios LE, Goheen MM, Bess LN, Watkins HS, Southern TR, et al. Antibody signature of spontaneous clearance of Chlamydia trachomatis ocular infection and partial resistance against re-challenge in a nonhuman primate trachoma model. PLoS Negl Trop Dis. Public Library of Science; 2013;7(5):e2248.

4. Wolle MA, Muñoz BE, Mkocha H, West SK. Constant ocular infection with Chlamydia trachomatis predicts risk of scarring in children in Tanzania. Ophthalmology. 2009 Mar;116(2):243–7.

5. West SK, Muñoz B, Mkocha H, Hsieh YH, Lynch MC. Progression of active trachoma to scarring in a cohort of Tanzanian children. Ophthalmic Epidemiol. 2001 Jul;8(2–3):137–44.

6. King J, Schindler C, Ngondi J, Odermatt P, Utzinger J, Mulualem A, et al. Impact of the SAFE strategy on trachomatous scarring among children in Ethiopia. In: Tropical Medicine and International Health, editor. Abstracts of the 9th European Congress on Tropical Medicine and International Health 6-10 September. Basel, Switzerland; 2015. p. 240.

7. Hu VH, Holland MJ, Burton MJ. Trachoma: protective and pathogenic ocular immune responses to Chlamydia trachomatis. PLoS Negl Trop Dis. 2013 Jan;7(2):e2020.

8. World Health Organization. Report of the 3rd Global Scientific Meeting on Trachoma. 19-20 July. Johns Hopkins University, Baltimore, MA; 2010.

9. Ramadhani AM, Derrick T, Macleod D, Holland MJ, Burton MJ. The Relationship between Active Trachoma and Ocular Chlamydia trachomatis Infection before and after Mass Antibiotic Treatment. Vinetz JM, editor. PLoS Negl Trop Dis. 2016 Oct 26;10(10):e0005080.

10. Butcher RMR, Sokana O, Jack K, Macleod CK, Marks ME, Kalae E, et al. Low Prevalence of Conjunctival Infection with Chlamydia trachomatis in a Treatment-Naïve Trachoma-Endemic Region of the Solomon Islands. PLoS Negl Trop Dis. 2016 Sep;10(9):e0004863.

11. Martin DL, Wiegand R, Goodhew B, Lammie P, Black CM, West S, et al. Serological Measures of Trachoma Transmission Intensity. Sci Rep. 2015 Jan;5:18532.

12. Kirkwood B, Sterne JA. Calculation of required sample size. In: Essentail Medical

Statistics. 2nd ed. Oxford, UK: Blackwell Publishing Ltd; 2003. p. 413–28.

13. Thylefors B, Dawson CR, Jones BR, West SK, Taylor HR. A simple system for the assessment of trachoma and its complications. Bull World Health Organ. 1987 Jan;65(4):477–83.

14. Solomon AW, Pavluck AL, Courtright P, Aboe A, Adamu L, Alemayehu W, et al. The Global Trachoma Mapping Project: Methodology of a 34-Country Population-Based Study. Ophthalmic Epidemiol. 2015 Jun;22(3):214–25.

15. Dawson CR, Jones BR, Tarizzo ML, World Health Organization. Guide to trachoma control in programmes for the prevention of blindness. 1981.

16. Solomon AW, Holland MJ, Burton MJ, West SK, Alexander ND, Aguirre A, et al. Strategies for control of trachoma: observational study with quantitative PCR. Lancet. 2003;362(9379):198–204.

17. Martin DL, Bid R, Sandi F, Goodhew EB, Massae PA, Lasway A, et al. Serology for Trachoma Surveillance after Cessation of Mass Drug Administration. PLoS Neglected Trop Dis [electronic Resour. 2015;9(2):e0003555.

18. Cocks N, Rainima-Qaniuci M, Yalen C, Macleod CK, Nakolinivalu A, Migchelsen S, et al. Community seroprevalence survey for yaws and trachoma in the Western Division of Fiji. Trans R Soc Trop Med Hyg. 2016;[manuscript in press].

19. Roberts CH, Last A, Molina-Gonzalez S, Cassama E, Butcher R, Nabicassa M, et al. Development and Evaluation of a Next-Generation Digital PCR Diagnostic Assay for Ocular Chlamydia trachomatis Infections. J Clin Microbiol. 2013 Jul;51(7):2195–203.

20. Macleod CK, Butcher R, Mudaliar U, Natutusau K, Pavluck AL, Willis R, et al. Low Prevalence of Ocular Chlamydia trachomatis Infection and Active Trachoma in the Western Division of Fiji. PLoS Negl Trop Dis. 2016 Jul;10(7):e0004798.

21. R Core Team. R: A Language and Environment for Statistical Computing [Internet]. R Foundation for Statistical Computing. 2014. Available from: http://www.r-project.org

22. Solomon Island Government. Report on 2009 population and housing census. 2011.

23. Marks M, Kako H, Butcher R, Lauri B, Puiahi E, Pitakaka R, et al. Prevalence of sexually transmitted infections in female clinic attendees in Honiara, Solomon Islands. BMJ Open. 2015;In Press.

24. Marks M, Kako H, Butcher R, Lauri B, Puiahi E, Pitakaka R, et al. Prevalence of sexually transmitted infections in female clinic attendees in Honiara, Solomon Islands. BMJ Open. 2015;5(4).

25. Schachter J, Holt J, Goodner E, Grossman M, Sweet R, Mills J. Propsective study of

chlamydial infection in neonates. Lancet. 1979;2(8139):377–80.

26. Goodhew EB, Morgan SM, Switzer AJ, Munoz B, Dize L, Gaydos C, et al. Longitudinal analysis of antibody responses to trachoma antigens before and after mass drug administration. BMC Infect Dis. 2014 Apr 22;14(1):216.

27. Goodhew EB, Priest JW, Moss DM, Zhong G, Munoz B, Mkocha H, et al. CT694 and pgp3 as serological tools for monitoring trachoma programs. PLoS Negl Trop Dis. 2012 Jan;6(11):e1873.

28. Horner P, Soldan K, Vieira SM, Wills GS, Woodhall SC, Pebody R, et al. C. trachomatis Pgp3 antibody prevalence in young women in England, 1993-2010. Trotter CL, editor. PLoS One. Public Library of Science; 2013 Jan;8(8):e72001.

29. Wolle MA, Muñoz B, Mkocha H, West SK. Age, sex, and cohort effects in a longitudinal study of trachomatous scarring. Invest Ophthalmol Vis Sci. 2009 Feb;50(2):592–6.

30. Faraj HG, Hoang-Xuan T. Chronic cicatrizing conjunctivitis. Curr Opin Ophthalmol. 2001;12(4):250–7.

31. Yohannan J, Munoz B, Mkocha H, Gaydos CA, Bailey R, Lietman TA, et al. Can we stop mass drug administration prior to 3 annual rounds in communities with low prevalence of trachoma?: PRET Ziada trial results. JAMA Ophthalmol. 2013 Apr;131(4):431–6.

32. Burton MJ, Holland MJ, Makalo P, Aryee EAN, Sillah A, Cohuet S, et al. Profound and sustained reduction in Chlamydia trachomatis in The Gambia: a five-year longitudinal study of trachoma endemic communities. Carvalho MS, editor. PLoS Negl Trop Dis. Public Library of Science; 2010 Jan;4(10):10.

33. Liu F, Porco TC, Amza A, Kadri B, Nassirou B, West SK, et al. Short-term Forecasting of the Prevalence of Trachoma: Expert Opinion, Statistical Regression, versus Transmission Models. PLoS Negl Trop Dis. 2015;9(8).

34. West SK, Munoz B, Weaver J, Mrango Z, Dize L, Gaydos C, et al. Can We Use Antibodies to Chlamydia trachomatis as a Surveillance Tool for National Trachoma Control Programs? Results from a District Survey. PLoS Negl Trop Dis. Public Library of Science; 2016 Jan 15;10(1):e0004352.

35. Marks M, Bottomley C, Tome H, Pitakaka R, Butcher R, Sokana O, et al. Mass drug administration of azithromycin for trachoma reduces the prevalence of genital Chlamydia trachomatis infection in the Solomon Islands. Sex Transm Infect. 2016 Feb 17;

36. Marks M, Vahi V, Sokana O, Chi K-HH, Puiahi E, Kilua G, et al. Impact of community mass treatment with azithromycin for trachoma elimination on the prevalence of yaws. PLoS Negl Trop Dis. 2015;9 (8) (no(8).

6.4 Note A: Exemplars of photographs graded for follicles, papillae and cicatricae

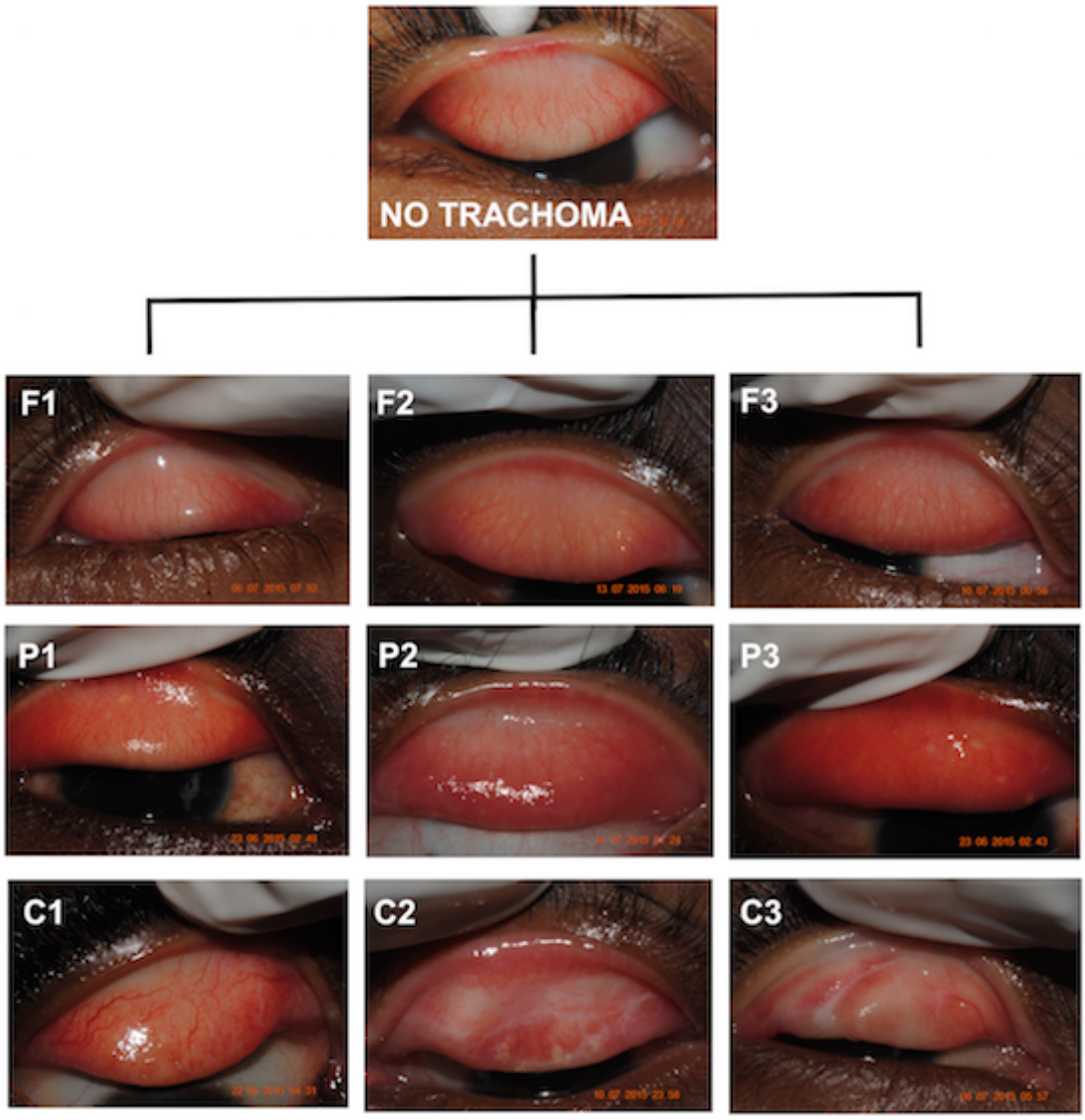


Figure 6.4.1. Exemplars of photographs graded as F, P and C. While the more severe grades are more reproducible, the milder grades can be subjective. Additionally, features not relevant for trachoma grading schemes are visible in some images, for example concretions visible in image C2 or light artefacts in image C1. The photograder can judge whether these are related to trachoma, but the assigned grade is based on the visible features, whether the grader thinks they are related to trachoma or not.

6.5 Note B: Scarring trachoma in a comparative population

The prevalence of conjunctival scars in these villages of the Solomon Islands increases with age, suggesting incident scars are still emerging. However, the proportion of older people with scarring is low, and the relative proportion of severe compared to mild scarring is similar throughout the age groups, suggesting progression is not taking place. This is consistent with the low levels of ocular infection observed. Mild scars are expected to start in childhood and progress to severe scars with age, but relatively few datasets have been generated which explore the process in detail as the most commonly used grading system is the simplified system, which doesn't differentiate between mild and severe scarring. A study conducted in Guinea Bissau in 2013 which graded all participants of any age in the field using the FPC system provided a useful comparator dataset with a high prevalence of *Ct* infection (26). The Solomon Island and Guinea Bissau studies were similar in size; approximately 1500 people of all ages living in randomly selected households in tropical island settings. The baseline unadjusted prevalence of TF/TI in 1–9 year olds in Guinea Bissau was 22% compared to 33.7% in the Solomon Islands. The prevalence of ocular *Ct* infection according to ddPCR in children aged 1–9 years in Guinea Bissau was 25%, whereas in the Solomon Islands the baseline prevalence of infection was 3.1% (26). Both studies utilised the FPC system. Figure 6.5.1 demonstrates the prevalence of severe scars in older age groups to be much lower in the Solomon Islands than in Guinea Bissau. This is consistent with the hypothesis in section 6.3 that progression of trachoma is relatively mild in the Solomon Islands compared to other trachoma-endemic regions. It is also evident that the prevalence of scars of any severity, and particularly eyelid-distorting scars, are more prevalent in the childhood age groups in Solomon Islands than in Guinea Bissau. This pattern of scars in children but not in adults could suggest that trachoma is increasing, and children are experiencing infectious stimuli that their parents have not. There is also likely to be an increased burden of neonatal conjunctivitis in the Solomon Islands due to the very high prevalence of sexually transmitted infections which can, in severe cases, lead to conjunctival scarring and entropion, therefore potentially inflating estimates of severe scarring from trachoma. However, as we demonstrate in figure 2 of section 6.3, those scars graded as C3 were noted upon grading not to look typically trachomatous due to significant sub-epithelial edema. This exposes a weakness of the grading system whereby distortion of the conjunctiva is graded as C3, even though the graders acknowledge the clinical presentation is not typical of trachoma.

These striking differences in age-specific scar prevalence are in populations with similarly striking differences in ocular *Ct* prevalence. Key distinctions between the studies should be considered as potential sources of bias when comparing these two plots. Firstly, the Guinea Bissau and Solomon Islands studies were carried out in populations that are likely to have very distinct genetic profiles, which is known to have an impact on scarring risk (263). The impact of host genetics on scarring risk is likely to be very complicated as described in the discussion of section 5.2, but may contribute to the discrepancy seen. Secondly, the Guinea Bissau data was

generated before MDA, whereas the Solomon Island data was generated 6 months after one round of MDA. That round of MDA 6 months previously is not likely to have altered our scarring prevalence estimates, as the process of scarring is not thought the be reversible and is thought to take years to develop; thus any reduction of scarring caused by MDA are likely to need longer to be observable (34). Thirdly, villages enrolled in the Guinea Bissau study were randomly selected, whereas in the Solomon Islands study villages were specifically selected for trachoma endemicity. This is unlikely to have led to an underestimation of age-specific scar prevalence in the Solomon Islands. Finally, the Guinea Bissau grades were generated in the field whereas in the Solomon Islands they were generated from photographs. Due to the two-dimensional nature of photographs, marginal cases of distortion of the conjunctiva (required to meet criteria for C3) are not always clear. Similarly, very fine scars may be more easily observed during clinical examination where the grader can adjust their position and the lighting on the conjunctiva to identify very fine changes to the texture or structure of the epithelial tissue. This is not possible from photographic examination. Both of these issues constitute limitations of photograph grading, which may be overcome using techniques that were unavailable during this study, such as *in vivo* confocal microscopy. However, in the case of these two comparator populations we do not expect those marginal cases to account for the gross differences in C3 prevalence between studies.

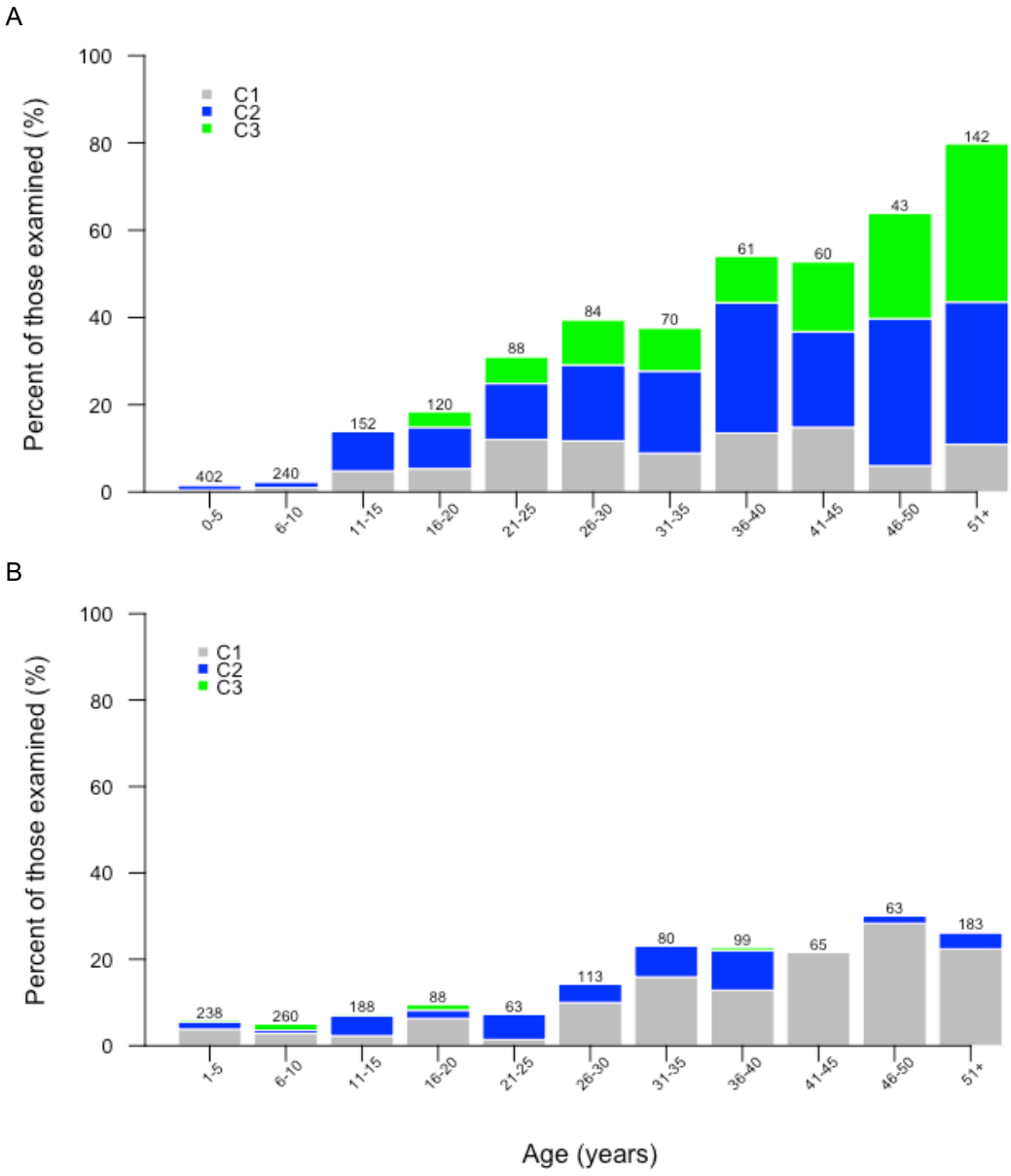


Figure 6.5.1. Age-specific prevalence of scarring in (A) Guinea Bissau (moderate trachomatous inflammation – follicular (TF), moderate ocular *Chlamydia trachomatis* (*Ct*) infection) and (B) the Solomon Islands (moderate TF, low ocular *Ct* infection). The numbers above each column represent the total number of people in each group. Height of bars shows total percentage of population with scars graded C1-C3. Colours indicate the proportion of each age group with C1 (grey), C2 (blue) and C3 (green) scars. Guinea Bissau fine scar grade data kindly shared by Anna Last.

6.6 Note C: Association between scarring and anti-Pgp3 antibody titre

Most adults in the study population were seropositive. However, the wide range of anti-Pgp3 antibody ODs and the significant increase in those over 18 years suggests a nontrachomatous origin to seropositivity which may confound estimates of relationship with scar grade (figure 6.7.1). It is therefore not appropriate to compare the Pgp3 level with scar grade in adults.

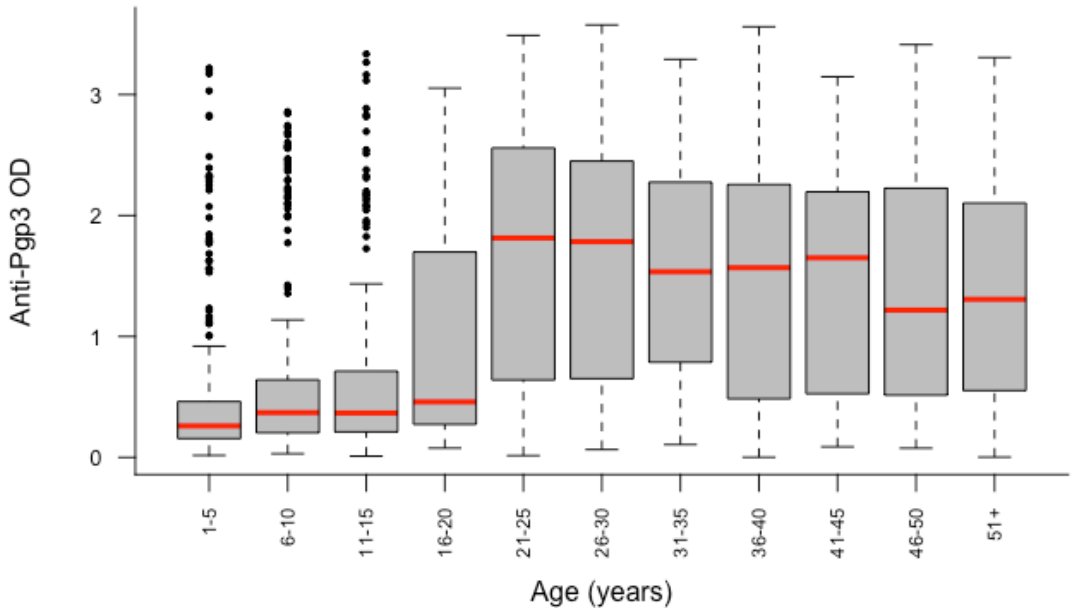


Figure 6.6.1. Normalised anti-Pgp3 optical density of specimens from Solomon Islanders of all ages showing wide range of responses in those aged over 18 years. Boxes illustrate inter-quartile range, and whiskers represent up to 1.5-times the inter-quartile range.

However, in children, ocular *Ct* should be the primary driver of Pgp3 seropositivity where it is present, therefore reducing the confounding effects of urogenital exposure. In the manuscript (section 6.3), a linear regression model of scar grade compared with Pgp3 level in children in the Solomon Islands is used to demonstrate that detectable levels of Pgp3 reactivity do not increase with scar grade. This is well illustrated by the normalised OD data from these children, as shown in figure 6.6.2. The anti-Pgp3 antibody level in children with conjunctival scarring in the Solomon Islands is no different to those without, suggesting no difference in prior exposure with *Ct.* This is further evidence that the scars in children, although unusually prevalent compared to areas with highly prevalent *Ct* infection (demonstrated in section 6.5), are unlikely to be a result of *Ct* infection.

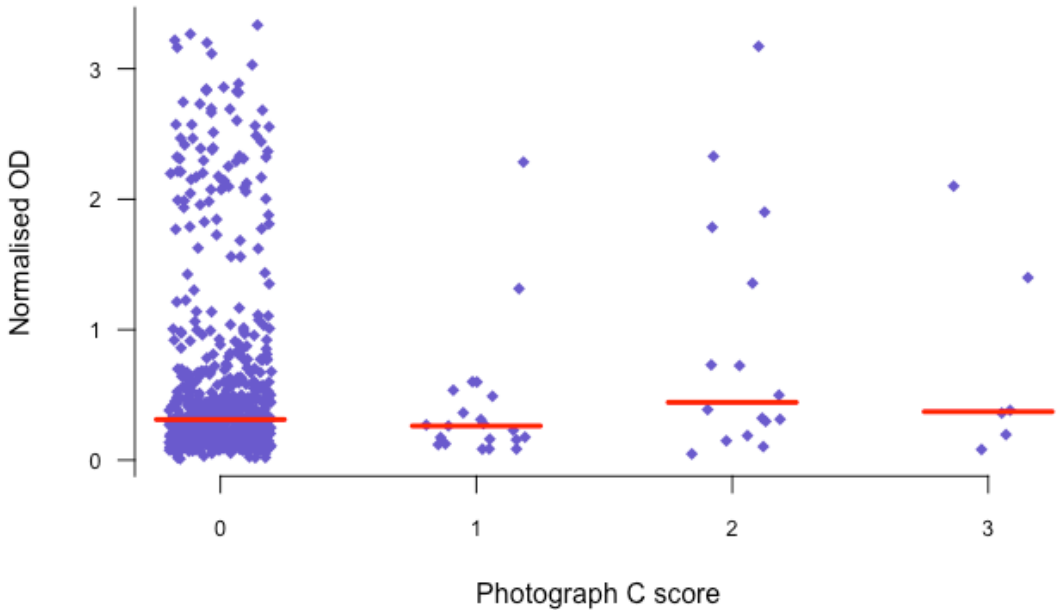


Figure 6.6.2. Relationship between optical density (OD) value and photograph C score in children aged 1–15 years. No difference in median Pgp3 level is observed between those with and those without scarring meeting the FPC grading system for trachoma. Red lines indicate median OD values for each scar grade.

7. AETIOLOGY OF TRACHOMATOUS INFLAMMATION – FOLLICULAR IN THE SOLOMON ISLANDS

7.1 Introduction

Internationally standardized mapping techniques demonstrated that the prevalence of clinical signs of trachoma in Melanesia is unusual. In the Solomon Islands, two independent measures of infection (ddPCR and ELISA) have indicated that the prevalence of *Ct* low, and is unlikely to explain the burden of TF. Antibody responses to *Ct* antigens, which are generated in response to ocular infection (239), were absent in most (80.3%) of the children with TF who were living in trachoma-endemic villages of the Solomon Islands. Furthermore, the prevalent conjunctivitis was not concurrent with severe scarring in adults, suggesting the chronic immunopathogenesis associated with repeated *Ct* infection may not be widespread in this population. A non-*Ct* cause for TF was suspected.

A number of potential candidate pathogens have been identified by the studies summarized in Table 1.7.2. Although numerous bacteria have been isolated from the conjunctivae of children with clinical signs of trachoma, *S. pneumoniae* (*Sp*) and *H. influenzae* (*Hi*) (both capsulated and noncapsulated subtypes) were the most likely suspects, as they have both been shown to correlate closely with TF in some populations.

In this chapter, I set out to determine whether common pathogens of the conjunctival, auricular and oral mucosae were associated with inflammation in this population. In section 6.3, the markers under investigation were long-lived and examining specimens from children after a round of MDA was appropriate. Infection, meanwhile, is transient and likely to be affected by MDA regardless of whether the organism itself is directly susceptible to azithromycin. Therefore, the pre-MDA specimen set described in chapter five was revisited and tested for the presence of several specific bacterial causes and one viral cause of conjunctivitis.

7.2 Manuscript

Whilst bacteria are detected in many (19%) of the specimens, none showed any statistically significant association with disease. A subset of these specimens was then tested using a less-targeted sequencing-based screening of poly-microbial communities, which has recently been applied to ocular specimens. Both the richness and diversity of bacteria at the conjunctiva are similar between those with visible follicular inflammation and those without. This was initially surprising given how different the mucosal environments must be at the molecular level, but other studies of diseased and nondiseased eyes have also failed to detect disease-associated shifts in microbiome (185,186).

These studies indicate that no bacterial species, genus or community structure appears to be sufficiently strongly linked to TF to explain the disease that was observed in the Solomon Islands. The final conclusion is that a nonbacterial cause is the most likely explanation of TF

and this further adds to the case against the continued use of TF as an indicator for MDA in the Solomon Islands.

RESEARCH PAPER COVER SHEET

SECTION A – Student Details

Student	*Robert Butcher*
Principal Supervisor	*Chrissy h Roberts*
Thesis Title	*Using alternate indicators of trachoma to estimate prevalence, characterise disease and define need for public health intervention: Evidence from the Pacific Islands*

If the Research Paper has previously been published, please complete Section B, if not please move to Section C

SECTION B – Paper already published

Where was the work published?	*NA*		
When was the work published?	*NA*		
If the work was published prior to registration for your research degree, give a brief rationale for its inclusion	*NA*		
Have you retained copyright for the work?*	*NA*	**Was the work subject to academic peer review?**	*NA*

If yes, please attach evidence of retention. If no, or if the work is being included in its published format, please attach evidence of permission from the copyright holder (publisher or other author) to include this work.* ***Work will be published under Creative Commons Attribution 4.0 International Open Access License.*

SECTION C – Prepared for publication, but not yet published

Where is the work intended to be published?	*Bulletin of the WHO*
Please list the paper's authors in the intended authorship order:	*Robert M R Butcher, Oliver Sokana, Kelvin Jack, Leslie Sui, Charles Russell, Joanna Houghton, Christine Palmer, Martin J Holland, Richard Le Mesurier, Anthony W Solomon, David CW Mabey, Chrissy h. Roberts.*
Stage of publication	*Under peer review*

SECTION D – Multi-authored work

For multi-authored work, give full details of your role in the research included in the paper and in the preparation of the paper.	*I supported Anthony Solomon in securing funding for this project. I worked with Anthony Solomon and Chrissy Roberts to obtain ethical approval for the study. The study was designed as part of the GTMP, I worked with Anthony to design the infection component. I helped Oliver, Kelvin, Eric, Leslie and Charles to conduct the fieldwork. I conducted the laboratory work. I analyzed the data with the help of Chrissy Roberts. I prepared the figures and tables, and wrote and revised the manuscript following feedback from co-authors.*

Student signature: _____ **Date:** 10/12/16

Supervisor signature: _____ **Date:** 19/12/16

Active trachoma cases in the Solomon Islands have varied polymicrobial community structures but do not associate with individual non-chlamydial pathogens of the eye.

Robert M R Butcher[1]*, Oliver Sokana[2], Kelvin Jack[2], Eric Kalae[3], Leslie Sui[3], Charles Russell[4], Joanna Houghton[1], Christine Palmer[1], Martin J Holland[1], Richard T Le Mesurier[5], Anthony W Solomon[1], David C W Mabey[1], Chrissy h. Roberts[1].

Author contact details:

[1] Clinical Research Department, London School of Hygiene & Tropical Medicine, Keppel Street, London WC1E 7HT, UK

[2] Eye Department, Solomon Island Ministry of Health and Medical Services, Honiara, Solomon Islands

[3] Primary Care Department, Lata Hospital, Lata, Santa Cruz, Solomon Islands

[4] Bellona Rural Health Centre, Bellona, Solomon Islands

[5] The Fred Hollows Foundation, Carlton, Victoria, Australia

*Corresponding author:

robert.butcher@lshtm.ac.uk, +44 207 927 2419

Keywords

Adenoviruses, droplet digital PCR, *Haemophilus influenzae, Moraxella catarrhalis,* Solomon Islands, *Staphylococcus aureus,* coagulase-negative *Staphylococcus, Streptococcus pneumoniae*, 16S rRNA gene sequencing, trachoma.

Abstract

Background

Several non-chlamydial microbial pathogens are associated with clinical signs of active trachoma in trachoma-endemic communities with a low prevalence of ocular *Chlamydia trachomatis* (*Ct*) infection. In the Solomon Islands, the prevalence of *Ct* among children is low despite the prevalence of active trachoma being moderate. We therefore set out to investigate whether active trachoma was associated with a common non-chlamydial infection or with a dominant polymicrobial community dysbiosis in the Solomon Islands.

Methods

We studied DNA from conjunctival swabs collected from 257 Solomon Islanders with active trachoma and matched controls. Droplet digital PCR was used to test for pathogens suspected to be able to induce follicular conjunctivitis. Polymicrobial community diversity and composition were studied by sequencing of hypervariable regions of the 16S ribosomal ribonucleic acid gene in a subset of 54 cases and 53 controls.

Results

Although *Ct* was associated with active trachoma, the number of infections was low (cases: 3.9%, controls: 0.4%). Estimated prevalence (cases, controls) of each non-chlamydial infection was as follows: *S. aureus* (1.9%, 1.9%), *Adenoviridae* (1.2%, 1.2%), coagulase-negative *Staphylococcus* (5.8%, 4.3%), *H. influenzae* (7.4%, 11.7%), *M. catarrhalis* (2.3%, 4.7%) and *S. pneumoniae* (7.0%, 6.2%). There was no statistically significant association between clinical signs of trachoma and presence or load of any of the non-*Ct* infections that were assayed. Inter-individual variations in the conjunctival microbiome were characterised by differences in the levels of *Corynebacterium*, *Proprionibacterium*, *Helicobacter* and *Paracoccus*, but diversity and relative abundance of these specific genera did not differ significantly between cases and controls.

Discussion

It is unlikely that the prevalent trachoma-like follicular conjunctivitis in the Solomon Islands has a dominant bacterial aetiology. Before implementing community-wide azithromycin distribution for trachoma, policy makers should consider that clinical signs of trachoma can be observed in the absence of any detectable azithromycin-susceptible organism.

Introduction

Trachoma, caused by *Chlamydia trachomatis* (*Ct*), is the leading infectious cause of preventable blindness (1), and is targeted for elimination as a public health problem by 2020 through the SAFE strategy (**S**urgery, **A**ntibiotics, **F**acial cleanliness and **E**nvironmental improvement). The decision to implement community-wide trachoma control interventions, which include mass drug administration (MDA) with azithromycin, is based on population prevalence estimates of one clinical sign of active trachoma (trachomatous inflammation–follicular [TF]) in the 1–9-year-old age group (2). In the Solomon Islands, the prevalence of TF is sufficient to warrant MDA but the prevalence of trachomatous trichiasis (TT) suggests trachoma may not pose a significant public health problem. A recent trachoma survey in a treatment-naïve population of the Solomon Islands estimated 26% of 1–9-year-olds to have TF but, surprisingly, conjunctival *Ct* infection was detected in only 1.3% of that age group (3). Whilst *Ct* infection is not always detectable in TF cases (4, 5), infection prevalence in the Solomon Islands is far lower than is seen in other countries with similar TF prevalence (3).

Ct is not the only pathogen to associate with signs of TF. A number of differential diagnoses for follicular conjunctivitis are described (6), some of which are rare and distinguishable by patient history (such as Parinaud's oculoglandular syndrome). Other causes of conjunctivitis which can present with follicles are *Streptococcus pneumoniae*, *Haemophilus influenzae* (7), and adenovirus (8). Non-ocular *Chlamydia* serotypes (9) and non-*trachomatis Chlamydia* species (10) have also been suggested as possible causes of TF. A number of studies have examined conjunctival microbiology in trachoma-endemic settings. A greater diversity of pathogens can be cultured from the conjunctivae of people with TF compared to counterparts without TF (7). Among bacterial species isolated during a study in Tanzanian children, *Streptococcus pneumoniae*, *Haemophilus influenzae* B and *Haemophilus influenzae* non-type B associated more strongly than *Ct* with clinical signs of active trachoma (7). In The Gambia, *S. pneumoniae* and *H. influenzae* type B infection correlated closely with signs of active trachoma in communities which had received MDA, whereas *Moraxella catarrhalis* and *Staphylococcus aureus* did not (11). Common non-chlamydial pathogens have associated with trachomatous scarring (TS) (12) and recurrence of TT after surgery (13) in some, but not all (14, 15), studies.

Although traditionally thought to be 'sterile', several studies have now described polymicrobial communities colonising the conjunctival epithelium (16, 17). *Staphylococcus*, *Streptococcus*, *Haemophilus* and *Moraxella* genera can be readily cultured from swabs taken from the inferior fornix (7, 11, 12). Attempts to detect bacteria from the conjunctiva have shown community diversity and composition to vary significantly between individuals. It is unclear whether a 'core' microbiota persists at the conjunctiva, but profiles closely related to that of the skin have been reported (18). *Corynebacterium*, *Propionibacterium*, *Staphylococcus* and *Streptococcus* have been consistently dominant, whereas other genera such as *Acinetobacter, Brevundimonas, Pseudomonas, Bradyrhizobium, Sphingomonas, Bacillus, Simonsiella* and *Elizabethkingia* have been identified more sporadically (19–21). Conjunctival polymicrobial community composition is

known to vary with age and season (22) and appears to be responsive to external stimuli such as regular contact lens wear (23), The conjunctival microbiome varies significantly between people with trachomatous scarring and those without, however, significant associations between microbiome and follicular conjunctival inflammation or microbiome and other diseases have yet to be described (22, 24).

We hypothesised that clinical signs of active trachoma in the Solomon Islands where ocular *Ct* is uncommon could be explained by a common non-chlamydial infection or by a dominant polymicrobial community dysbiosis.

Methods

Study ethics

Ethical approval was granted by the London School of Hygiene & Tropical Medicine (6319/6360) and the Solomon Islands National Health Research (HRC13/18) Ethics Committees. A parent or guardian provided written consent for each child participant.

Study population

The samples tested during this study were a sub-set of specimens from a population-based trachoma prevalence survey of 3674 people (1135 children aged 1–9 years) in 32 clusters from Temotu and Rennell & Bellona Provinces, Solomon Islands; data from that survey are presented elsewhere (3). At the time of the survey, no trachoma interventions had been implemented in the Solomon Islands. Sterile swabs were passed three times over the right conjunctiva of every child before storage in 300 μL RNALater® (Life Technologies). Swabs were kept cool in the field for up to 24hrs then frozen (3). Inclusion criteria for cases (n = 257) were age 1–9 years, detectable human DNA in the conjunctival swab and a field grade of TF or trachomatous inflammation—intense (TI) in the right eye. An equal number (n = 257) of age-, gender- and island-matched controls without TF or TI were selected using the 'e1071' R package. A random subset of cases (n = 54) and controls (n = 53) were further characterized using V1-V3 16S rRNA gene sequencing. Field controls (clean swabs passed within 20 cm of seated participants, without touching them, and then treated identically to subjects' specimens), extraction controls (extraction carried out in the absence of a specimen and PCR carried out on the eluate) and PCR controls (PCR carried out in the absence of any added material) were used to determine background levels of microbial contamination in the 16S rRNA gene sequencing protocol.

Droplet digital PCR

Note A - page 176

DNA was extracted from conjunctival swabs using the Qiagen AllPrep DNA/RNA kit. We chose not to use mechanical lysis on these low biomass specimens due to the reported lower yields compared to chemical lysis (25). All specimens have previously been tested for *Ct* and *Homo sapiens* Ribonuclease P protein subunit p30 (RPP30, acting as endogenous control) using a validated assay (26); the community prevalence of ocular *Ct* infection is described elsewhere (3).

Duplex ddPCR assays were developed for (1) *Adenoviridae* (27) and *S. pneumoniae* (28), (2) *H. influenzae* (29) and *M. catarrhalis* (30, 31), and (3) *S. aureus* and coagulase-negative *Staphylococcus* (32, 33) based on published assays. Each assay contained 10 μL 2× ddPCR Supermix for probes (Bio-rad, Hemel Hempstead, UK), primers and probes at custom concentrations (Table 1) and 8 μL of template DNA. Thermal cycling was 10'00'' at 95°C then

40x (0’15’’ at 95°C | 1’00’’ at 60°C) then 12’00’’ at 98°C (26). A positive result for a clinical specimen was defined as >95% confidence of non-zero target load, as described previously (26). Assay performance was assessed by repeat testing of PCR product dilution series’ and cultured pathogen material before rolling out to clinical samples. The reproducibility, linearity and limits of detection of each assay were considered appropriate (Supplementary Table 1) and were also similar to published performance of the *Ct* assay used (26).

16S rRNA gene sequencing

An approximately 530-bp region of the 16S ribosomal RNA (rRNA) gene (variable regions 1-3) was amplified using forward (modified 27F; 5'-[adaptor]-AGAGTTTGGATCCTGGCTCAG-3') and custom barcoded reverse primers (534R; 5'-[adaptor]-[barcode]-AGTCAGTCAGCCATTACCGCGGCTGCTGG-3'). Each 15 µL reaction contained 7.5 µL 2x Phusion High Fidelity Master Mix (New England Biosciences, MA, USA), 0.45 µL DMSO, 0.1 µM primers and 5.55 µL DNA. The thermal cycling conditions were 0’30’’ at 98°C then 31x (0’10’’ at 98°C | 0’30’’ at 62°C | 0’15’’ at 72°C) then 7’00’’ at 72°C. Amplicons were cleaned using 0.6 v/v AMPure XP beads (Beckman Coulter, CA, USA), quantified using Qubit (Thermo Fisher Scientific, MA, USA) then pooled at equimolar concentrations. The 4 nM sequencing library was mixed 0.75 v/v with a 4 nM Phi-X control library (Illumina, CA, USA). 3 µL of 100 µM custom read primers were mixed in to wells 12 (Read 1; CTACACTATGGTAATTGTAGAGTTTGGATCCTGGCTCAG), 13 (Index; CCAGCAGCCGCGGTAATGGCTGACTGACT) and 14 (Read 2; AGTCAGTCAGCCATTACCGCGGCTGCTGG) of the MiSeq reagents cartridge before 2 x 300 bp paired-end sequencing with the 600-cycle MiSeq v3 sequencing reagent kit on the MiSeq platform using a standard protocol (Illumina, CA, USA). 96 uniquely barcoded specimens were run in multiplex on each MiSeq run.

Data analysis

ddPCR data were analysed using R version 3.2.2 (34). Binomial univariate regression was used to test the relationship between each individual infection and active trachoma. The most accurate final multivariate model, determined by lowest Akaike Information Criterion (AIC) value, was determined by step-wise removal of variables from a binomial multivariate regression model incorporating all tested pathogens. The chance of the *Ct* association occurring due to chance was assessed by counting the number of significant results obtained from randomly re-ordering the *Ct* data 1000 times.

Raw 16S amplicon sequences were directly assigned to genera using Illumina BaseSpace ‘16S Metagenomics’ app version 1.0.1.0, which uses algorithms from Wang and colleagues (35). Two clinical specimens yielded fewer than 1000 reads in total and were removed from the analysis. Amplicons were sequenced from six no-template control (NTC) specimens (two ‘field’ controls, two ‘extraction’ controls, two ‘PCR’ controls, defined above) to identify any

contaminants endogenous to the collection, PCR or sequencing process. Any genera represented by more than 600 reads in the six combined NTCs (average 100 reads per NTC) were eliminated from the clinical specimen analysis. This resulted in the removal of 21 genera, which accounted for 95.1% of the reads from NTCs and 88.3% of the reads from clinical specimens. The genera removed included common contaminants of reagent kits (36) such as *Pelomonas*, as well as some previously described conjunctival microbiota constituents such as *Brevundimonas*, *Staphylococcus* and *Streptococcus* (19, 22).

Between-group differences in the Shannon Diversity Index (a measure of diversity which increases with increasing species abundance and evenness) and Inverse Simpson Index (another measure of diversity where 1 is infinite diversity and 0 is no diversity) were compared using a t-test. Discriminant Analysis of Principal Components (DAPC), a multivariate clustering method to analyse highly dimensional datasets, was performed using the 'adegenet' package in R (37) and cross-validation was used to determine the optimal number of principal components to include in a discriminant function aimed at separating cases from controls on the basis of their polymicrobial community structures.

Results

Specimen set demographics

A total of 257 cases and 257 controls (n = 514) were tested. All 257 cases had TF, and one also had TI. Case status was defined by clinical signs in the right eye but respectively 236/257 (91.8%) and 8/257 (3.1%) of the cases and controls had TF in the left eye. Both case and control groups were 38% female. Mean age was 5.6 years (cases) and 5.5 years (controls, student's t test p = 0.76). There was no significant difference between cases and controls in terms of the clusters represented (Kolmogorov-Smirnov test p = 0.97) and all 32 clusters were represented. The case specimens had higher loads of human DNA than the controls (18030 versus 9354 copies/swab; p = 0.00003). There were no significant differences in age, gender or location within the subset selected for 16S-amplicon sequencing.

Quantitative PCR tests for ocular pathogens

In this study, 19.8% of children had evidence of infection with at least one of the targeted organisms (Table 2). We considered the prevalence of *Ct* in this sample set to be too low (96.1% of TF cases were *Ct*-infection negative) to account for the level of active disease. The prevalence of *Adenoviridae* (1.2%) and *S. aureus* (1.9%) were both very low. There was no association between active trachoma and infection with *H. influenzae, S. pneumoniae, S. aureus,* coagulase-negative *Staphylococcus* or *M. catarrhalis. Ct* infection was associated with active trachoma (logistic regression p = 0.026; odds ratio: 10.4). The association between *Ct* and active trachoma was still significant in a multivariate analysis (p = 0.025). The permuted p-

value for the association between *Ct* and active trachoma was highly significant (p = 0.001). Active disease was neither associated with infection with at least one pathogen (any pathogen, p = 0.185) nor the number of concurrent infections in the same eye (number of concurrent pathogens, p = 0.207). Infection loads of those who tested positive are shown Figure 1. Despite some numerical differences between groups, none of the differences were statistically significant.

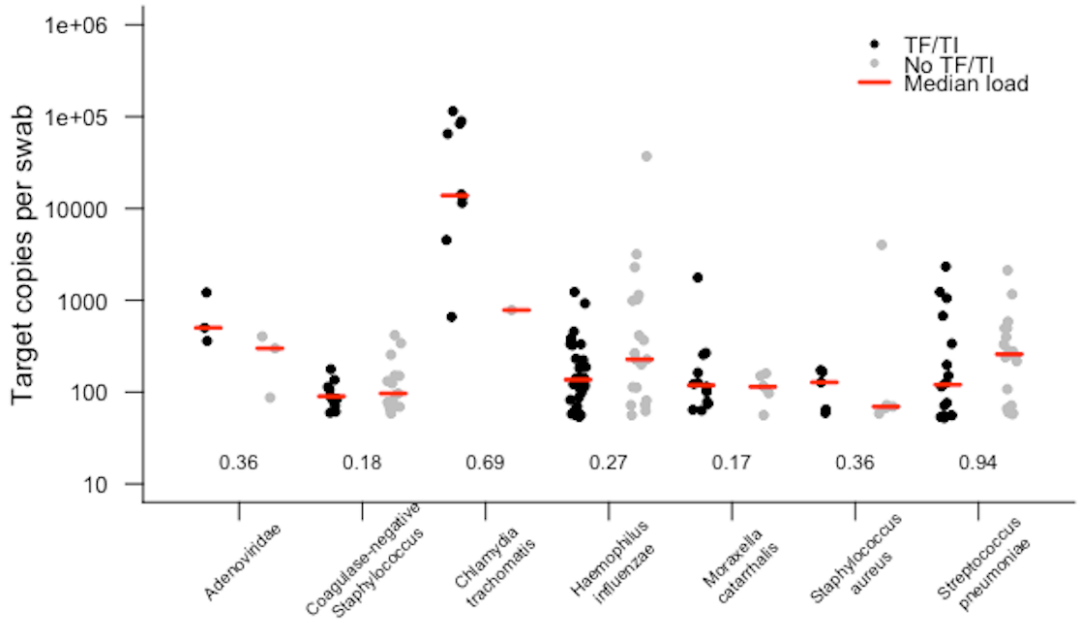


Figure 1. Target copies per swab of each pathogen identified in conjunctival swabs collected from children with and without active trachoma in the Solomon Islands. Numbers show p-values for logistic regression comparison between active trachoma case and control groups for each pathogen. None of the differences observed were statistically significant.

16S rRNA gene sequencing

Note B - page 182

The total number of genera identified across all clinical specimens and NTCs was 659, however, 125/659 (19%) had a cumulative total of ten reads or fewer. The median number of reads per clinical specimen was 55,104 (IQR: 40,705 – 89,541) and the median percentage of reads mapped to genus level was 51.9%. The median number of genera identified was 151 (IQR: 117 – 212) per clinical specimen. Following removal of presumed contaminants, approximately 40% of cleaned reads were assigned to genera that each constituted less than 1% of the total polymicrobial community. 40 genera were represented by at least 1% of remaining reads in clinical samples.

The diversity of bacterial communities was in general low; the mean Inverse Simpsons Diversity index over all specimens was 0.061 (IQR: 0.046 – 0.092). The median Inverse Simpson's Diversity index in cases was 0.061 [IQR: 0.048 – 0.095] versus controls 0.060 [IQR: 0.044 – 0.078] (Student's t-test p = 0.56). The median Shannon Diversity Index was 3.38 in cases and 3.37 in controls (Student's t-test p = 0.96). There was no significant difference in alpha-diversity

between cases and controls by either measure of diversity. In cases of active trachoma, the most dominant bacterial genera were *Corynebacterium* (12.0% of total reads), *Proprionibacterium* (6.2%) and *Helicobacter* (4.8%). In controls, the most dominant genera were *Corynebacterium* (13.9%), *Paracoccus* (5.2%), *Proprionibacterium* (4.7%), and *Neisseria* (4.1%) (Supplementary Figure 1). The genus level membership of the bacterial community varied between cases and controls. Of 21 genera found in specimens from those with active trachoma, 10 were not found in controls, including *Helicobacter*, *Mesoplasma, Brachybacterium* and *Haemophilus*. Of 23 genera found in controls, 12 were not found in cases, including *Neisseria, Prevotella, Rhodococcus* and *Porphyromonas*.

Principal Components (PC) Analysis (Figure 2, Supplementary Figure 2) revealed that *Corynebacterium* (PC1), *Paracoccus* (PC2), *Propionibacterium* (PC2, PC3) and *Helicobacter* (PC3) are major contributors to the variation in the conjunctival microbiomes of children in the Solomon Islands. While individual cases or controls have distinctive profiles dominated by these genera, the majority of cases and controls are indistinguishable (Figure 2) using the first three PCs (explaining 45% of total variation). We condensed PCs 1-20 into a single discriminant function, which explained 87% of variation in the data. In this analysis, the genus-level polymicrobial community structure is significantly different between cases and controls (logistic regression $p = 0.0000062$) (Figure 3). That discriminant function was dominated by a number of genera such as *Curvibacterium* and *Mesoplasma* (Supplementary Figure 3). Cross validation to predict group membership using discriminant functions of between 10 and 90 PCs had a low success rate (median 53.3% success, range: 50.1 – 62.3%). This suggests that while differences do exist between the polymicrobial communities in cases and controls, they are too subtle and varied to be predictive of phenotype, at least when working with this number of specimens.

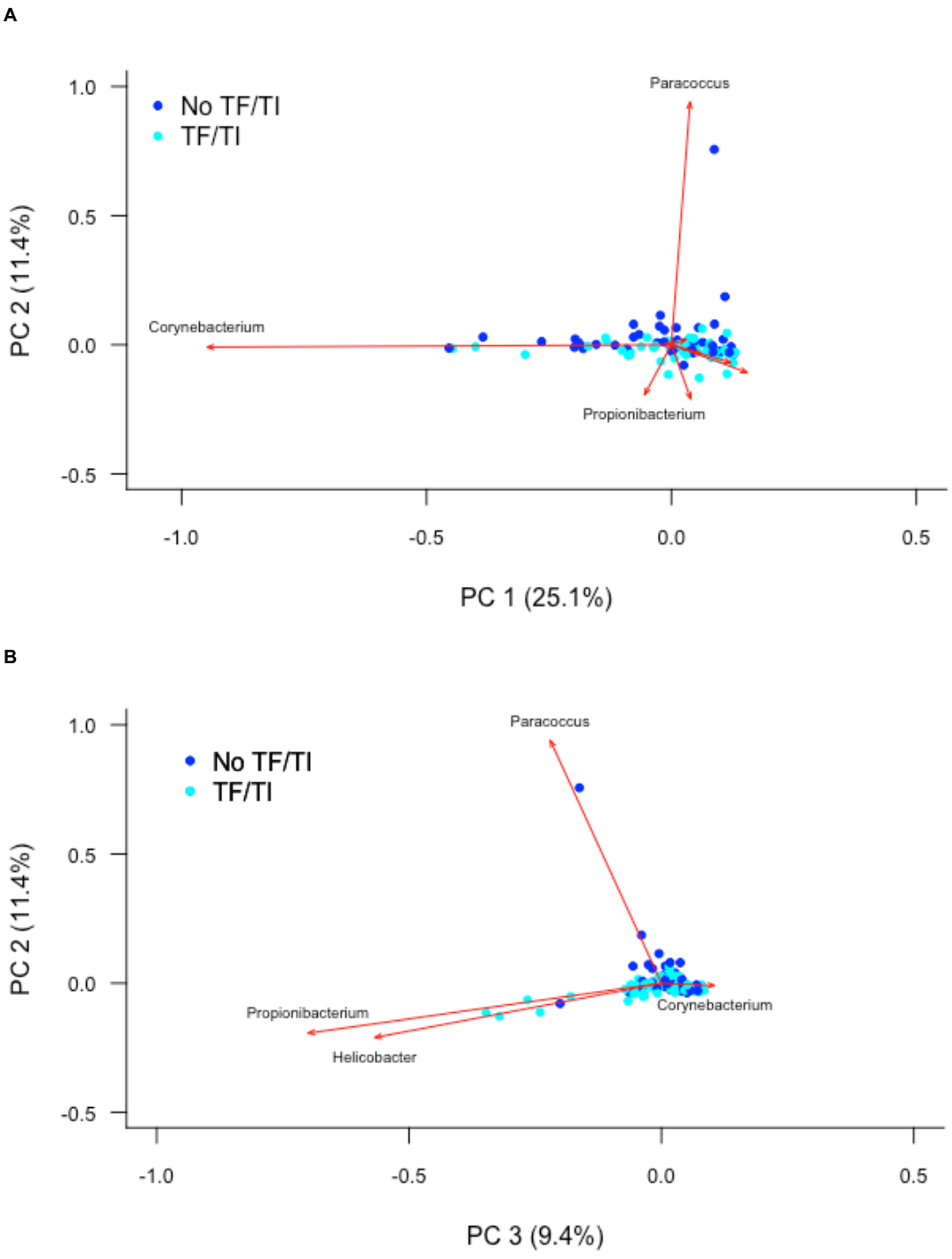


Figure 2. (A) First and second and **(B)** second and third principal components describing variation between the 16S sequences identified in Solomon Island children with and without active trachoma. Dark blue spots indicate controls. Light blue spots indicate cases. Red arrows show principal component loadings.

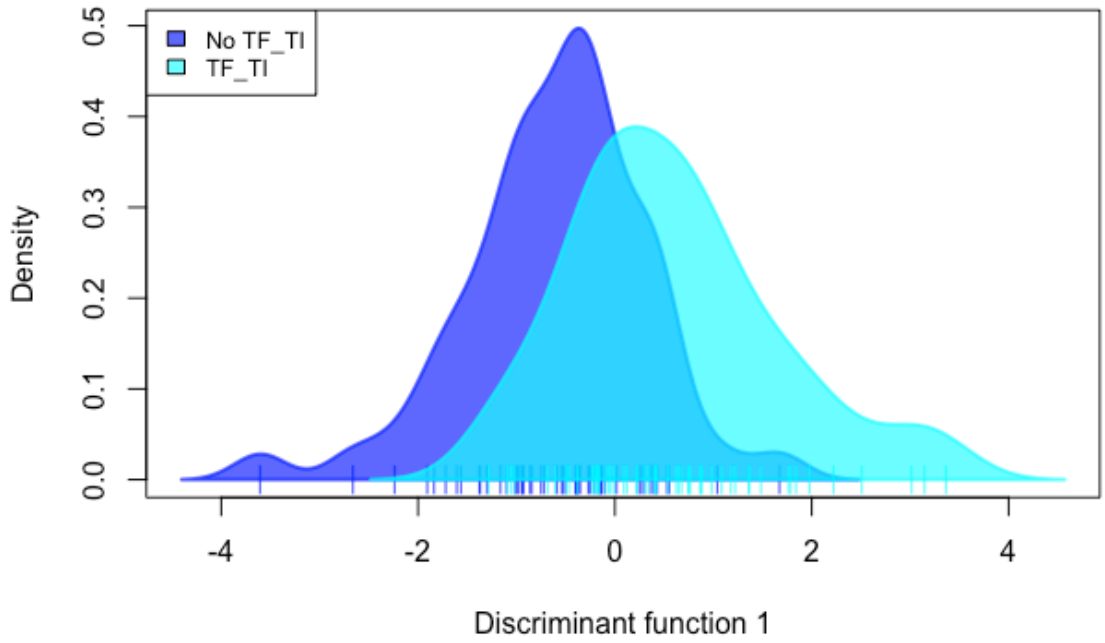


Figure 3: Discriminant analysis of the association of 20 combined principal components with active trachoma, showing a significant discrimination of phenotype groups (p = 0.000006).

Discussion

Follicular conjunctivitis meeting criteria for the trachoma phenotype TF is highly prevalent in the Solomon Islands, but the prevalence of *Ct* infection is curiously low. Although *Ct* was the only pathogen to associate with TF in this study, the prevalence was much lower than may be expected of a population with this level of TF (3). Both *S. pneumoniae* and *H. influenzae* were detected at moderate prevalence in our population, whilst we found very few cases of *Adenoviridae* and *M. catarrhalis* infection. Comparator data from other populations are scarce, but it is clear that staphylococcal species were detected in the conjunctivae in the Solomon Islands at a substantially lower prevalence (7%) than in children in either Tanzania (14.8% *S. epidermidis*) (7), Sierra Leone (20% *S. aureus,* 29% coagulase-negative *Staphylococcus*) (38) or The Gambia (post MDA, 14.7% *S. aureus*) (11). By ddPCR testing for a number of bacteria and viruses that have previously been linked to TF, we can discount the possibility that any of them can account either singly or *en masse* for the high TF prevalence.

Through 16S amplicon sequencing and community profiling, we have shown that there is apparently no dominant bacterial genus associated with this disease. The dominant features of variation in conjunctival bacterial communities in Solomon Islands children (*Corynebacterium, Propionibacterium)* have consistently been identified in other studies. Other genera (e.g., *Paracoccus*, *Helicobacter, Haemophilus)* have not previously been identified in 16S studies, whilst some reported in other studies (e.g., *Simonsella, Pseudomonas*) were not found in these specimens (19–22). Many studies have sequenced the V3-4 region of the 16S gene whereas we targeted V1-3. V region choice has been shown to have an impact on the genera identified at other mucosal sites (39) and, while no data have yet emerged on how this affects profiles from the eye, it is possible this could account for some of the differences. Consistent with previous studies (36), we found a background of genera that amplified in NTCs that also appeared in clinical specimens. The microbiological biomass at the conjunctiva is known to be very low, even compared with other nearby sites such as skin or oral mucosa (20). When true resident bacteria are scarce, 16S rRNA gene PCR readily amplifies reagent contaminants. We took stringent measures to exclude reagent contaminants, but this resulted in the removal of some important genera such as *Staphylococcus* and *Streptococcus.* Among the biggest contributors to between-conjunctiva variation were *Corynebacterium* and *Propionibacterium*, but these genera did not differentiate TF cases from controls (Figure 2). Previous investigations of the role of the conjunctival microbiome in ocular disease have also not shown significant differences. In The Gambia, there was an increased abundance of *Haemophilus* in cases of TF, as compared to controls, although this difference was not significant (22). People with ocular manifestations of rosacea, Sjögren's syndrome and healthy controls did not have significant differences in diversity or relative abundance of key phyla identified across all three groups (24). However, other disease associations have been identified. For example, the abundance of *Corynebacterium* and *Streptococcus* varied significantly between individuals with and without conjunctival scarring (22).

The microbiota of these children were heterogeneous and had subtle variations that appeared to reach significant association with TF when the full community structure was considered in comparative statistical models. It remains possible (though perhaps unlikely) that a multitude of factors, operating at the level of single bacterial species, polymicrobial communities or viral species, all contribute to the presentation of the phenotype. It is perhaps more likely that as yet unidentified viral or allergic causes could explain the prevalence of TF in the Solomon Islands.

There are some limitations to our study. Firstly, although the ddPCR assays are based on validated assays and appear to be accurate (Supplementary Table 1), they have not been formally evaluated in this format. Secondly, by not using mechanical lysis in our extraction process, the absolute prevalence of some difficult-to-lyse Gram positive genera (e.g., *Staphylococcus*) may have been under-estimated. This would not impair the comparison of cases to controls, between which protocols were consistent. Thirdly, 16S amplicon sequencing of low biomass samples is known to result in amplification of reagent and environmental contaminants (36). This study focused on gross differences in community structure between those with and without disease and these should be independent of contaminating reads. We also employed stringent quality control measures to ensure data were not confused by artefacts of the sequencing process.

Given the current international commitment to trachoma elimination, further characterisation of the role of non-*Ct* stimuli in TF is important. We might expect those with TF in the absence of *Ct* to be at lower risk of progression to TS and TT than those with repeated *Ct* infection and inflammation (40), however, such individuals have not been studied longitudinally to assess outcomes therefore it is unclear how these cases should be managed. Furthermore, years after MDA has been administered, TF persists at levels above the threshold for elimination (>5%) in communities where *Ct* infection is not readily detectable (41, 42). The public health risk of trachoma in these communities is also unclear.

Public health scenarios such as the one in the Solomon Islands will become increasingly common as trachoma elimination programmes reduce the global prevalence of *Ct,* and the positive predictive value of TF for ocular Ct infection declines as a result (43). MDA might be inappropriately delivered when clinical signs of trachoma are the only indicator used for programmatic decision making. While MDA is effective for the treatment of trachoma (44), mass antibiotic exposure may increase macrolide resistance in nonchlamydial bacteria (45) and theoretically could make the population more susceptible to later infections by preventing accumulation of acquired immunity to *Ct* (46). Decisions to undertake such a program should therefore be carefully considered. The case for using tests for *Ct* infection during trachoma surveys is strengthened by our data. The value of nucleic acid amplification tests for detecting non-chlamydial infection remains questionable, as multiple infectious agents might be important, and these are likely to differ between populations.

Acknowledgements

We thank Eric Kalae, Leslie Sui (Primary Care Department, Lata Hospital, Solomon Islands) and Charles Russell (Bellona Rural Health Centre, Solomon Islands) for their help conducting the study fieldwork; Victoria Miari and Emma Cobb (Pathogen Molecular Biology Department, London School of Hygiene & Tropical Medicine, UK) for providing bacterial culture isolates for PCR assay validation; and David Nelson (Indiana University, Indianapolis, US) for the provision of 16S sequencing primers and protocols. We would also like to thank the residents of the communities who took part in this study.

Financial statement

Fieldwork was jointly funded by the United Kingdom's Department for International Development Global Trachoma Mapping Project grant (ARIES: 203145) to Sightsavers, and by the Fred Hollows Foundation, Australia (1041). Laboratory costs were funded by the Fred Hollows Foundation, Australia (1041).

RMRB and AWS were funded by a Wellcome Trust Intermediate Fellowship to AWS (098521).

OS and KJ were employed by the Solomon Islands Ministry of Health and Medical Services for the duration of the survey.

MJH is supported by the Wellcome Trust (093368/Z/10/Z).

ChR is supported by the Wellcome Trust Institutional Strategic Support Fund (105609/Z/14/Z).

Author Contributions

Conceived and designed the study: RMRB, OS, RTLM, AWS, DCWM, ChR.

Performed the fieldwork: RMRB, OS, KJ

Performed the experiments: RMRB, MJH, JH, CP, ChR.

Analysed the data: RMRB, ChR.

Wrote the manuscript: RMRB, ChR.

Revised and approved the manuscript: RMRB, OS, KJ, JH, CP, MJH, RTLM, AWS, DCWM, ChR.

References

1. Bourne RA, Stevens GA, White RA, Smith JL, Flaxman SR, Price H, Jonas JB, Keeffe J, Leasher J, Naidoo K, Pesudovs K, Resnikoff S, Taylor HR, Vision Loss Expert G. 2013. Causes of vision loss worldwide, 1990-2010: a systematic analysis. Lancet Glob Heal 1:e339-49.
2. World Health Organization. 2003. Report of the 2nd Global Scientific Meeting on Trachoma. 25-27 August. Geneva, Switzerland.
3. Butcher RMR, Sokana O, Jack K, Macleod CK, Marks ME, Kalae E, Sui L, Russell C, Tutill HJ, Williams RJ, Breuer J, Willis R, Le Mesurier RT, Mabey DCW, Solomon AW, Roberts CH. 2016. Low Prevalence of Conjunctival Infection with Chlamydia trachomatis in a Treatment-Naïve Trachoma-Endemic Region of the Solomon Islands. PLoS Negl Trop Dis 10:e0004863.
4. Thein J, Zhao PN, Liu HS, Xu JJ, Jha HC, Miao YH, Pizzarello LD, Tapert L, Schachter J, Mabon M, Osaki-Holm S, Lietman T, Paxton A. 2002. Does clinical diagnosis indicate ocular chlamydial infection in areas with a low prevalence of trachoma? Ophthalmic Epidemiol 9:263–269.
5. Grassly NC, Ward ME, Ferris S, Mabey DC, Bailey RL. 2008. The natural history of trachoma infection and disease in a Gambian cohort with frequent follow-up. PLoS Negl Trop Dis 2:e341.
6. Dawson CR, Jones BR, Tarizzo ML, World Health Organization. 1981. Guide to trachoma control in programmes for the prevention of blindness. Geneva, Switzerland: World Health Organization.
7. Burton MJ, Hu VH, Massae P, Burr SE, Chevallier C, Afwamba IA, Courtright P, Weiss HA, Mabey DCW, Bailey RL. 2011. What is causing active trachoma? The role of nonchlamydial bacterial pathogens in a low prevalence setting. Invest Ophthalmol Vis Sci 52:6012–7.
8. Jhanji V, Chan TCYY, Li EYMM, Agarwal K, Vajpayee RB. 2015. Adenoviral keratoconjunctivitis. Surv Ophthalmol 60:435–443.
9. Andersson P, Harris SR, Smith HMBS, Hadfield J, O'Neill C, Cutcliffe LT, Douglas FP, Asche LV, Mathews JD, Hutton SI, Sarovich DS, Tong SYC, Clarke IN, Thomson NR, Giffard PM. 2016. Chlamydia trachomatis from Australian Aboriginal people with trachoma are polyphyletic composed of multiple distinctive lineages. Nat Commun 7:10688.
10. Lietman T, Brooks D, Moncada J, Schachter J, Dawson C, Dean D. 1998. Chronic follicular conjunctivitis associated with Chlamydia psittaci or Chlamydia pneumoniae. Clin Infect Dis 26:1335–40.
11. Burr SE, Hart JD, Edwards T, Baldeh I, Bojang E, Harding-Esch EM, Holland MJ, Lietman TM, West SK, Mabey DCW, Sillah A, Bailey RL. 2013. Association between ocular bacterial carriage and follicular trachoma following mass azithromycin distribution in The Gambia. PLoS Negl Trop Dis 7:e2347.
12. Hu VH, Massae P, Weiss HA, Chevallier C, Onyango JJ, Afwamba IA, Mabey DCW,

Bailey RL, Burton MJ. 2011. Bacterial infection in scarring trachoma. Invest Ophthalmol Vis Sci 52:2181–6.

13. Burton MJ, Adegbola RA, Kinteh F, Ikumapayi UN, Foster A, Mabey DCW, Bailey RL. 2007. Bacterial infection and trachoma in the gambia: a case control study. Invest Ophthalmol Vis Sci 48:4440–4.
14. Cox JT, Kasubi MJ, Mu BE, Zambrano AI, Greene GS, Mkocha H, Wolle MA, West SK. 2017. Trachomatous Scarring and Infection With Non–Chlamydia Trachomatis Bacteria in wmoen in Kongwa, Tanzania. Investig Ophthalmol Vis Sci 58:3249–3253.
15. Burton MJ, Adegbola RA, Kinteh F, Ikumapayi UN, Foster A, Mabey DCW, Bailey RL. 2007. Bacterial infection and trachoma in the gambia: A case-control study. Invest Ophthalmol Vis Sci 48:4440–4444.
16. Kugadas A, Gadjeva M. 2016. Impact of Microbiome on Ocular Health. Ocul Surf 14:342–9.
17. Lu LJ, Liu J. 2016. Focus: Microbiome: Human Microbiota and Ophthalmic Disease. Yale J Biol Med 89:325.
18. Zhou Y, Gao H, Mihindukulasuriya KA, La Rosa PS, Wylie KM, Vishnivetskaya T, Podar M, Warner B, Tarr PI, Nelson DE, Fortenberry JD, Holland MJ, Burr SE, Shannon WD, Sodergren E, Weinstock GM. 2013. Biogeography of the ecosystems of the healthy human body. Genome Biol 14:R1.
19. Dong Q, Brulc JM, Iovieno A, Bates B, Garoutte A, Miller D, Revanna K V, Gao X, Antonopoulos DA, Slepak VZ, Shestopalov VI. 2011. Diversity of bacteria at healthy human conjunctiva. Invest Ophthalmol Vis Sci 52:5408–13.
20. Doan T, Akileswaran L, Andersen D, Johnson B, Ko N, Shrestha A, Shestopalov V, Lee CS, Lee AY, Van Gelder RN. 2016. Paucibacterial Microbiome and Resident DNA Virome of the Healthy Conjunctiva. Investig Opthalmology Vis Sci 57:5116.
21. Huang Y, Yang B, Li W. 2016. Defining the normal core microbiome of conjunctival microbial communities. Clin Microbiol Infect 22:643.e7-643.e12.
22. Zhou Y, Holland MJ, Makalo P, Joof H, Roberts CH, Mabey D, Bailey RL, Burton MJ, Weinstock GM, Burr SE. 2014. The conjunctival microbiome in health and trachomatous disease: a case control study. Genome Med 6:99.
23. Shin H, Price K, Albert L, Dodick J, Park L, Dominguez-Belloa MG. 2016. Changes in the eye microbiota associated with contact lens wearing. MBio 7:1–6.
24. de Paiva CS, Jones DB, Stern ME, Bian F, Moore QL, Corbiere S, Streckfus CF, Hutchinson DS, Ajami NJ, Petrosino JF, Pflugfelder SC. 2016. Altered Mucosal Microbiome Diversity and Disease Severity in Sjögren Syndrome. Sci Rep 6:23561.
25. Abusleme L, Hong B-Y, Dupuy AK, Strausbaugh LD, Diaz PI. 2014. Influence of DNA extraction on oral microbial profiles obtained via 16S rRNA gene sequencing. J Oral Microbiol 6.
26. Roberts CH, Last A, Molina-Gonzalez S, Cassama E, Butcher R, Nabicassa M, McCarthy E, Burr SE, Mabey DC, Bailey RL, Holland MJ. 2013. Development and Evaluation of a Next-Generation Digital PCR Diagnostic Assay for Ocular Chlamydia trachomatis Infections. J Clin Microbiol 51:2195–203.

27. Heim A, Ebnet C, Harste G, Pring-Akerblom P. 2003. Rapid and quantitative detection of human adenovirus DNA by real-time PCR. J Med Virol 70:228–39.
28. Carvalho M da GS, Tondella ML, McCaustland K, Weidlich L, McGee L, Mayer LW, Steigerwalt A, Whaley M, Facklam RR, Fields B, Carlone G, Ades EW, Dagan R, Sampson JS. 2007. Evaluation and improvement of real-time PCR assays targeting lytA, ply, and psaA genes for detection of pneumococcal DNA. J Clin Microbiol 45:2460–6.
29. Meyler KL, Meehan M, Bennett D, Cunney R, Cafferkey M. 2012. Development of a diagnostic real-time polymerase chain reaction assay for the detection of invasive Haemophilus influenzae in clinical samples. Diagn Microbiol Infect Dis 74:356–62.
30. Greiner O, Day PJR, Altwegg M, Nadal D. 2003. Quantitative detection of Moraxella catarrhalis in nasopharyngeal secretions by real-time PCR. J Clin Microbiol 41:1386–90.
31. Kais M, Spindler C, Kalin M, Ortqvist A, Giske CG. 2006. Quantitative detection of Streptococcus pneumoniae, Haemophilus influenzae, and Moraxella catarrhalis in lower respiratory tract samples by real-time PCR. Diagn Microbiol Infect Dis 55:169–78.
32. Nakagawa S, Taneike I, Mimura D, Iwakura N, Nakayama T, Emura T, Kitatsuji M, Fujimoto A, Yamamoto T. 2005. Gene sequences and specific detection for Panton-Valentine leukocidin. Biochem Biophys Res Commun 328:995–1002.
33. Okolie CE, Wooldridge KG, Turner DP, Cockayne A, James R. 2015. Development of a new pentaplex real-time PCR assay for the identification of poly-microbial specimens containing Staphylococcus aureus and other staphylococci, with simultaneous detection of staphylococcal virulence and methicillin resistance markers. Mol Cell Probes 29:144–50.
34. R Core Team. 2014. R: A Language and Environment for Statistical Computing. R Found Stat Comput.
35. Wang Q, Garrity GM, Tiedje JM, Cole JR. 2007. Naive Bayesian Classifier for Rapid Assignment of rRNA Sequences into the New Bacterial Taxonomy. Appl Environ Microbiol 73:5261–5267.
36. Salter SJ, Cox MJ, Turek EM, Calus ST, Cookson WO, Moffatt MF, Turner P, Parkhill J, Loman NJ, Walker AW, Kunin V, Engelbrektson A, Ochman H, Hugenholtz P, Fv W, Göbel U, Stackebrandt E, Kulakov L, McAlister M, Ogden K, Larkin M, O'Hanlon J, McAlister M, Kulakov L, O'Hanlon J, Larkin M, Ogden K, Kéki Z, Grébner K, Bohus V, Márialigeti K, Tóth E, Bohus V, Kéki Z, Márialigeti K, Baranyi K, Patek G, Schunk J, Tóth E, McFeters G, Broadaway S, Pyle B, Egozy Y, Nogami T, Ohto T, Kawaguchi O, Zaitsu Y, Sasaki S, Shen H, Rogelj S, Kieft T, Rand K, Houck H, Maiwald M, Ditton H, Sonntag H, Doeberitz M von K, Tanner M, Goebel B, Dojka M, Pace N, Corless C, Guiver M, Borrow R, Edwards-Jones V, Kaczmarski E, Fox A, Grahn N, Olofsson M, Ellnebo-Svedlund K, Monstein H, Jonasson J, Newsome T, Li B, Zou N, Lo S, Mohammadi T, Reesink H, Vandenbroucke-Grauls C, Savelkoul P, Barton H, Taylor N, Lubbers B, Pemberton A, Laurence M, Hatzis C, Brash D, Oberauner L, Zachow C, Lackner S, Högenauer C, Smolle K, Berg G, Duc M La, Kern R, Venkateswaran K, Ling Z, Liu X, Luo Y, Yuan L, Nelson K, Wang Y, Xiang C, Li L, Benítez-Páez A, Álvarez M, Belda-Ferre P, Rubido S, Mira A, Tom I, Amar J, Serino M, Lange C, Chabo C, Iacovoni J,

Mondot S, Lepage P, Klopp C, Mariette J, Bouchez O, Perez L, Courtney M, Marre M, Klopp P, Lantieri O, Doré J, Charles M, Balkau B, Burcelin R, Branton W, Ellestad K, Maingat F, Wheatley B, Rud E, Warren R, Holt R, Surette M, Power C, Borewicz K, Pragman A, Kim H, Hertz M, Wendt C, Isaacson R, Dong Q, Brulc J, Iovieno A, Bates B, Garoutte A, Miller D, Revanna K, Gao X, Antonopoulos D, Slepak V, Shestopalov V, Xuan C, Shamonki J, Chung A, DiNome M, Chung M, Sieling P, Lee D, Kuehn J, Gorden P, Munro D, Rong R, Dong Q, Plummer P, Wang C, Phillips G, Srinivas G, Möller S, Wang J, Künzel S, Zillikens D, Baines J, Ibrahim S, Boissière A, Tchioffo M, Bachar D, Abate L, Marie A, Nsango S, Shahbazkia H, Awono-Ambene P, Levashina E, Christen R, Morlais I, McKenzie V, Bowers R, Fierer N, Knight R, Lauber C, Carlos C, Torres T, Ottoboni L, Cheng X, Tian X, Wang Y, Lin R, Mao Z, Chen N, Xie B, Davidson S, Powell R, James S, Knowlton C, Veerapaneni R, D'Elia T, Rogers S, Shtarkman Y, Koçer Z, Edgar R, Veerapaneni R, D'Elia T, Morris P, Rogers S, DeLeon-Rodriguez N, Lathem T, Rodriguez-R L, Barazesh J, Anderson B, Beyersdorf A, Ziemba L, Bergin M, Nenes A, Konstantinidis K, Turner P, Turner C, Jankhot A, Helen N, Lee S, Day N, White N, Nosten F, Goldblatt D, Willerslev E, Hansen A, Poinar H, Kearney M, Spindler J, Wiegand A, Shao W, Anderson E, Maldarelli F, Ruscetti F, Mellors J, Hughes S, Grice S Le, Coffin J, Cooper A, Poinar H, Roberts C, Ingham S, Segal L, Alekseyenko A, Clemente J, Kulkarni R, Wu B, Chen H, Berger K, Goldring R, Rom W, Blaser M, Weiden M, Bhatt A, Freeman S, Herrera A, Pedamallu C, Gevers D, Duke F, Jung J, Michaud M, Walker B, Young S, Earl A, Kostic A, Ojesina A, Hasserjian R, Ballen K, Chen Y, Hobbs G, Antin J, Soiffer R, Baden L, Garrett W, Hornick J, Marty F, Meyerson M, Deragon J, Sinnett D, Mitchell G, Potier M, Labuda D, Sarkar G, Sommer S, Klaschik S, Lehmann L, Raadts A, Hoeft A, Stuber F, Tamariz J, Voynarovska K, Prinz M, Caragine T, Hilali F, Saulnier P, Chachaty E, Andremont A, Heininger A, Binder M, Ellinger A, Botzenhart K, Unertl K, Döring G, Silkie S, Tolcher M, Nelson K, Carroll N, Adamson P, Okhravi N, Mohammadi T, Reesink H, Vandenbroucke-Grauls C, Savelkoul P, Hughes M, Beck L, Skuce R, Vaishampayan P, Probst A, Duc M La, Bargoma E, Benardini J, Andersen G, Venkateswaran K, Rueckert A, Morgan H, Patel P, Garson J, Tettmar K, Ancliff S, McDonald C, Pitt T, Coelho J, Tedder R, Champlot S, Berthelot C, Pruvost M, Bennett E, Grange T, Geigl E, Chang S, Hsu H, Cheng J, Tseng C, Bonfert T, Csaba G, Zimmer R, Friedel C, Knights D, Kuczynski J, Charlson E, Zaneveld J, Mozer M, Collman R, Bushman F, Knight R, Kelley S, Eren A, Maignen L, Sul W, Murphy L, Grim S, Morrison H, Sogin M, Morris A, Beck J, Schloss P, Campbell T, Crothers K, Curtis J, Flores S, Fontenot A, Ghedin E, Huang L, Jablonski K, Kleerup E, Lynch S, Sodergren E, Twigg H, Young V, Bassis C, Venkataraman A, Schmidt T, Weinstock G, Lane D, Klindworth A, Pruesse E, Schweer T, Peplies J, Quast C, Horn M, Gl°Ckner F, Cooper P, Walker A, Reyes J, Chico M, Salter S, Vaca M, Parkhill J, Schloss P, Westcott S, Ryabin T, Hall J, Hartmann M, Hollister E, Lesniewski R, Oakley B, Parks D, Robinson C, Sahl J, Stres B, Thallinger G, Horn D Van, Weber C, Kozich J, Westcott S, Baxter N, Highlander S, Schloss P, Quince C, Lanzen A, Davenport R, Turnbaugh P, Bolger A, Lohse M, Usadel B, Kiełbasa S, Wan R, Sato K, Horton P, Frith

M, Huson D, Mitra S, Ruscheweyh H, Weber N, Schuster S, Schloss P, Gevers D, Westcott S, White J, Nagarajan N, Pop M. 2014. Reagent and laboratory contamination can critically impact sequence-based microbiome analyses. BMC Biol 12:87.

37. Jombart T, Devillard S, Balloux F, Falush D, Stephens M, Pritchard J, Pritchard J, Stephens M, Donnelly P, Corander J, Waldmann P, Sillanpaa M, Tang J, Hanage W, Fraser C, Corander J, Lee C, Abdool A, Huang C, Jombart T, Jombart T, Devillard S, Dufour A, Pontier D, Jombart T, Pontier D, Dufour A, McVean G, Novembre J, Stephens M, Patterson N, Price A, Reich D, Price A, Patterson N, Plenge R, Weinblatt M, Shadick N, Reich D, Hotelling H, Hotelling H, Pearson K, Liu N, Zhao H, Fisher R, Lachenbruch P, Goldstein M, Aitchison J, Reyment R, Beharav A, Nevo E, Fraley C, Raftery A, Cann H, Toma C de, Cazes L, Legrand M, Morel V, Piouffre L, Bodmer J, Bodmer W, Bonne-Tamir B, Cambon-Thomsen A, Ramachandran S, Deshpande O, Roseman C, Rosenberg N, Feldman M, Cavalli-Sforza L, Rosenberg N, Pritchard J, Weber J, Cann H, Kidd K, Zhivotovsky L, Feldman M, Wang S, Lewis C, Jakobsson M, Ramachandran S, Ray N, Bedoya G, Rojas W, Parra M, Molina J, Gallo C, Balloux F, Rosenberg N, Mahajan S, Ramachandran S, Zhao C, Pritchard J, Feldman M, Rambaut A, Pybus O, Nelson M, Viboud C, Taubenberger J, Holmes E, Russell C, Jones T, Barr I, Cox N, Garten R, Gregory V, Gust I, Hampson A, Hay A, Hurt A, Smith D, Lapedes A, Jong J de, Bestebroer T, Rimmelzwaan G, Osterhaus A, Fouchier R, Holmes E, Ghedin E, Miller N, Taylor J, Bao Y, George KS, Grenfell B, Salzberg S, Fraser C, Lipman D, Young J, Palese P, Benson D, Karsch-Mizrachi A, Lipman D, Ostell J, Wheeler D, Larkin M, Blackshields G, Brown N, Chenna R, McGettigan P, McWilliam H, Valentin F, Wallace I, Wilm A, Lopez R, Waterhouse A, Procter J, Martin D, Clamp M, Barton G, Paradis E, Claude J, Strimmer K, Handley L, Manica A, Goudet J, Balloux F, Serre D, Paabo S, Corander J, Marttinen P, Siren J, Tang J, Francois O, Ancelet S, Guillot G, Hunley K, Healy M, Long J, Kittles R, Weiss K, Manica A, Prugnolle F, Balloux F, Prugnolle F, Manica A, Balloux F, Romero I, Manica A, Handley L, Balloux F, Amos W, Hoffman J, Fraley C, Raftery A, Peres-Neto P, Jackson D, Somers K, Saporta G, Paradis E, Dray S, Dufour A, Schwarz G, Evanno G, Regnaut S, Goudet J, Jakobsson M, Rosenberg N, Chessel D, Dufour A, Thioulouse J, Dray S, Dufour A, Chessel D, Venables W, Ripley B, Nei M. 2010. Discriminant analysis of principal components: a new method for the analysis of genetically structured populations. BMC Genet 11:94.

38. Capriotti JA, Pelletier JS, Shah M, Caivano DM, Ritterband DC. 2009. Normal ocular flora in healthy eyes from a rural population in Sierra Leone. Int Ophthalmol 29:81–4.

39. Walker AW, Martin JC, Scott P, Parkhill J, Flint HJ, Scott KP. 2015. 16S rRNA gene-based profiling of the human infant gut microbiota is strongly influenced by sample processing and PCR primer choice. Microbiome 3:26.

40. Wolle MA, Muñoz BE, Mkocha H, West SK. 2009. Constant ocular infection with Chlamydia trachomatis predicts risk of scarring in children in Tanzania. Ophthalmology 116:243–7.

41. Martin DL, Bid R, Sandi F, Goodhew EB, Massae PA, Lasway A, Philippin H, Makupa W, Molina S, Holland MJ, Mabey DCW, Drakeley C, Lammie PJ, Solomon AW. 2015.

Serology for Trachoma Surveillance after Cessation of Mass Drug Administration. PLoS Negl Trop Dis 9:e0003555.

42. Bird M, Dawson CR, Schachter JS, Miao Y, Shama A, Osman A, Bassem A, Lietman TM. 2003. Does the diagnosis of trachoma adequately identify ocular chlamydial infection in trachoma-endemic areas? J Infect Dis 187:1669–73.
43. Ramadhani AM, Derrick T, Macleod D, Holland MJ, Burton MJ. 2016. The Relationship between Active Trachoma and Ocular Chlamydia trachomatis Infection before and after Mass Antibiotic Treatment. PLoS Negl Trop Dis 10.
44. Solomon AW, Holland MJ, Alexander NDE, Massae PA, Aguirre A, Natividad-Sancho A, Molina S, Safari S, Shao JF, Courtright P, Peeling RW, West SK, Bailey RL, Foster A, Mabey DCW. 2004. Mass treatment with single-dose azithromycin for trachoma. N Engl J Med 351:1962–1971.
45. Seidman JC, Coles CL, Silbergeld EK, Levens J, Mkocha H, Johnson LB, Muñoz B, West SK, Munoz B, West SK. 2014. Increased carriage of macrolide-resistant fecal E. coli following mass distribution of azithromycin for trachoma control. Int J Epidemiol 43:1105–1113.
46. Brunham RC, Rekart ML. 2008. The Arrested Immunity Hypothesis and the Epidemiology of Chlamydia Control. Sex Transm Dis 35:53–54.
47. Last AR, Roberts CH, Cassama E, Nabicassa M, Molina-Gonzalez S, Burr SE, Mabey DCW, Bailey RL, Holland MJ. 2013. Plasmid copy number and disease severity in naturally occurring ocular Chlamydia trachomatis infection. J Clin Microbiol 52:324.

Table 1. Oligonucleotides used in this study, with assay concentrations derived from *in vitro* optimisation.

Organism / target	Oligo	Sequence (5'-3')	Concentration in final assay (nM)	Amplicon size	Ref
C. trachomatis / Plasmid open reading frame 2 (*pORF2*)	F	CAG CTT GTA GTC CTG CTT GAG AGA	900	109	(3, 26, 47)
	R	CAA GAG TAC ATC GGT CAA CGA AGA	900		
	Probe	[FAM] CCC CAC CAT TTT TCC GGA GCG A [BHQ1]	200		
S. aureus / Staphylococcal protein A (*SpA*)	F	CAG CAA ACC ATG CAG ATG CTA	900	101	(32, 33)
	R	CGC TAA TGA TAA TCC ACC AAA TAC A	900		
	Probe	[VIC] TCA AGC ATT ACC AGA AAC [MGBNFQ]	250		
Coagulase-negative *Staphylococcus* / translation elongation factor (*tuf*)	F	TAT CCA CGA AAC TTC TAA AAC AAC TGT TAC T	450	204	(33)
	R	TCT TTA GAT AAT ACG TAT ACT TCA GCT TTG AAT TT	450		
	Probe	[FAM] TAT TAG ACT ACG CTG AAG CTG GTG ACA ACA T [BHQ1]	125		
S. pneumoniae / N-acetylmuramoyl-L-alanine amidase (*lytA*)	F	ACG CAA TCT AGC AGA TGA AGC A	500	75	(28)
	R	TCG TGC GTT TTA ATT CCA GCT	500		
	Probe	[FAM] GCC GAA AAC GCT TGA TAC AGG GAG [BHQ1]	150		
H. influenzae / L-fuculokinase (*fucK*)[†]	F	ATG GCG GGA ACA TCA ATG A	900	102	(29)
	R	ACG CAT AGG AGG GAA ATG GTT	900		
	Probe*	[FAM] CGg TAa TTg GGa TCc AT [BHQ1]	125		
Adenoviridae / capsid assembly protein (hexon)	F	GCC ACG GTG GGG TTT CTA AAC TT	500	132	(27)
	R	GCC CCA GTG GTC TTA CAT GCA CAT C	500		
	Probe	[HEX] TGC ACC AGA CCC GGG CTC AGG TAC TCC GA [BHQ1]	125		
M. catarrhalis / outer membrane protein (*copB*)	F	GTG AGT GCC GCT TTT ACA ACC	900	72	(30, 31)
	R	TGT ATC GCC TGC CAA GAC AA	900		
	Probe	[HEX] TGC TTT TGC AGC TGT TAG CCA GCC TAA [BHQ1]	250		

* Lower case symbols denote locked nucleic acid bases

[†] These primers do not differentiate between encapsulated and noncapsulated subtype

Table 2. Cases of infection in specimens from children with and without active disease. The relationship of those infections to trachoma has been tested with univariate logistic regression, and then stepwise removal of variables from multivariate regression model was used to determine the final multivariate regression model that provided the best fit for the data.

Pathogen	ddPCR result	No TF/TI (%; n=257)	TF/TI (%; n=257)	Total (%; n=514)	Univariate odds ratio (95% CI)	Univariate p-value	Multivariate odds ratio (95% CI)	Multivariate p-value
Adenoviridae	Positive	3 (1.2)	3 (1.2)	6 (1.2)	1.00 (0.67 – 1.50)	1.000	-	-
	Negative	254 (98.8)	254 (98.8)	508 (98.8)				
*Chlamydia trachomatis**	Positive	1 (0.4)	10 (3.9)	11 (2.1)	10.36 (1.96 – 190.92)	**0.026**	10.64 (2.15 – 196.00)	**0.025**
	Negative	256 (99.6)	247 (96.1)	503 (97.9)				
Coagulase-negative *Staphylococcus*	Positive	15 (5.8)	11 (4.3)	26 (5.1)	0.72 (0.32 – 1.59)	0.423	-	-
	Negative	242 (94.2)	246 (95.7)	488 (94.9)				
Haemophilus influenzae	Positive	19 (7.4)	30 (11.7)	49 (9.5)	1.66 (0.91 – 3.07)	0.101	-	-
	Negative	238 (92.6)	227 (88.3)	465 (90.4)				
Moraxella catarrhalis	Positive	6 (2.3)	12 (4.7)	18 (3.5)	2.05 (0.78 – 5.96)	0.158	2.13 (0.81 – 6.20)	0.137
	Negative	251 (97.7)	245 (95.3)	496 (96.5)				
Staphylococcus aureus	Positive	5 (1.9)	5 (1.9)	10 (1.9)	1.00 (0.28 – 3.64)	1.000	-	-
	Negative	252 (98.1)	252 (98.1)	504 (98.1)				
Streptococcus pneumoniae	Positive	18 (7.0)	16 (6.2)	34 (6.6)	0.88 (0.43 – 1.77)	0.723	-	-
	Negative	239 (93.0)	241 (93.8)	480 (93.4)				
Any pathogen	Positive	45 (17.5)	57 (22.2)	102 (19.8)	0.75 (0.48 – 1.15)	0.185	Not included	
	Negative	212 (82.4)	200 (77.8)	412 (80.2)				
Different pathogen species in same eye	0	212 (82.4)	200 (77.8)	412 (80.2)	1.21 (0.90 – 1.63)	0.207	Not included	
	1	28 (10.9)	35 (13.6)	63 (12.3)				
	>1	17 (6.6)	22 (8.6)	39 (7.6)				

*Data taken from Butcher *et al.*(3)

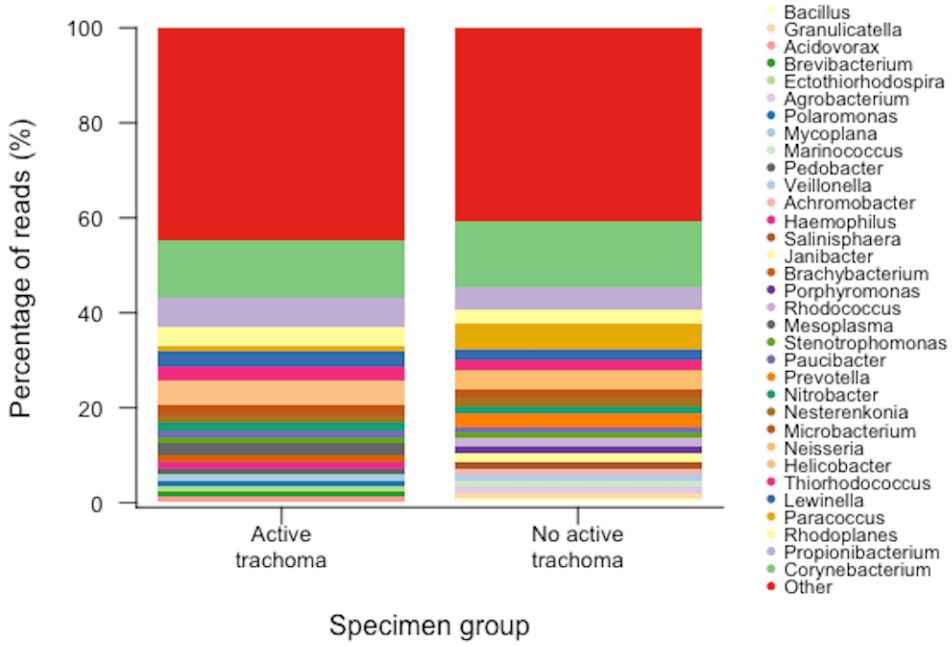
Supplementary information

Supplementary table 1: Each PCR product for dilution into standards was prepared using TaqMan Universal II PCR mix (Life Technologies, Paisley, UK), cleaned with Qiagen MinElute PCR product kit (Qiagen, Manchester, UK) and serially diluted from $1:10^6$ to $1:10^{12}$ in ten-fold dilution steps. Each series was tested in five technical replicates with duplex ddPCR assays to determine the reproducibility of the assay. Target concentrations between one and 10 copies/µL were reproducibly detected by all six assays. The coefficient of determination for all assays was in excess of 0.99 when fitted to a linear regression model. All six assays were highly reproducible, and the mean coefficient of variance (CoV) was 12.1% (range: 7.8 – 14.7%).

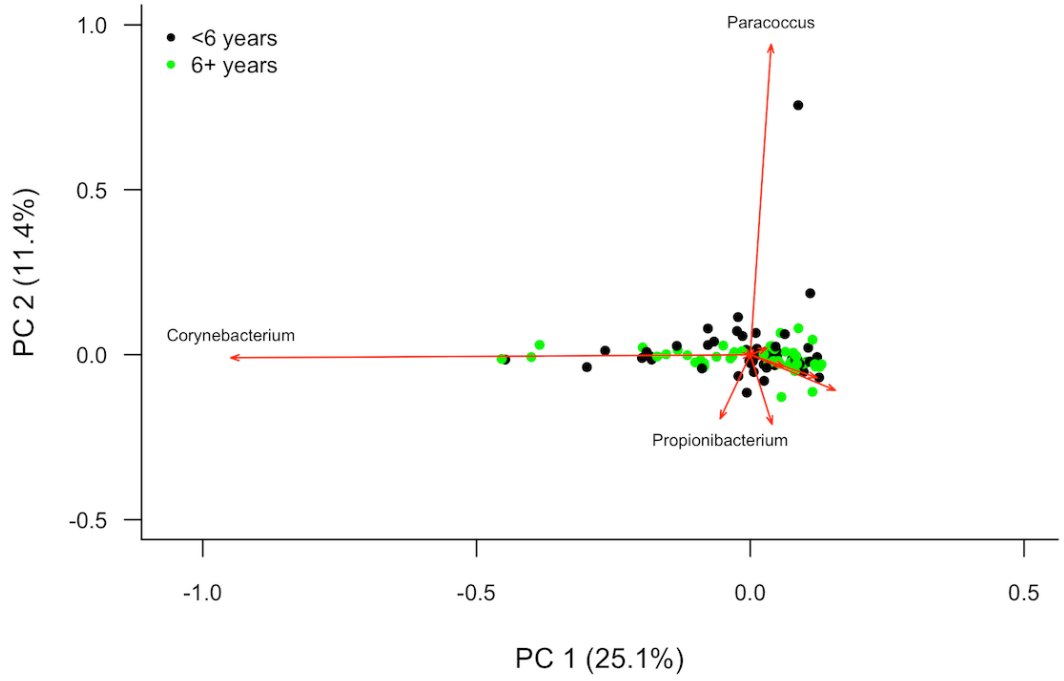
Assay	Standard curve *		
	LoD	R^2	CoV (%)
S. aureus	4.0	0.991	14.4
Coagulase-negative *Staphylococcus*	1.2	0.994	14.7
S. pneumoniae	9.9	0.998	11.9
H. influenza	2.3	0.997	14.7
Adenoviridae	3.0	0.997	9.1
M. catarrhalis	1.0	0.993	7.8

CoV: Coefficient of variation; LoD: Limit of detection: R^2: Coefficient of determination

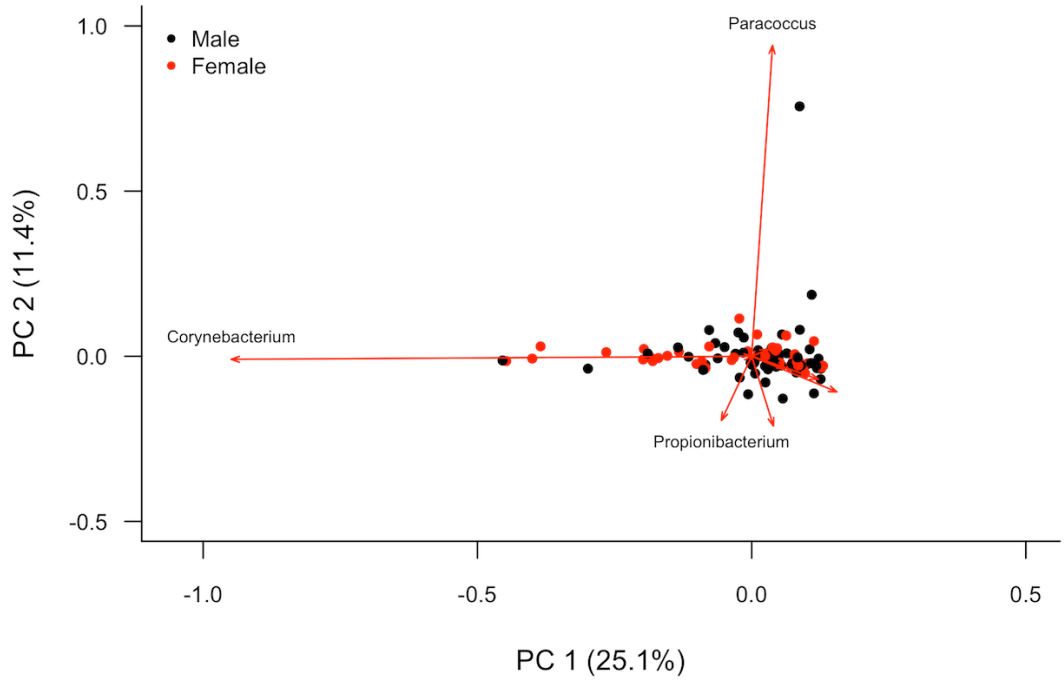
* Values calculated from 10-fold dilution series of PCR product between approximately 10^5 and 10^1 copies per target per test with 5 technical replicates at each dilution point.



Supplementary figure 1. Relative taxa abundance in age-, sex- and location-matched children with TF/TI (n = 54) and without (n = 53). Relative taxa abundance is expressed as percentage of total reads per specimen group. Genera with reads representing less than 1% of the total number of reads were combined into a group entitled 'Other'.

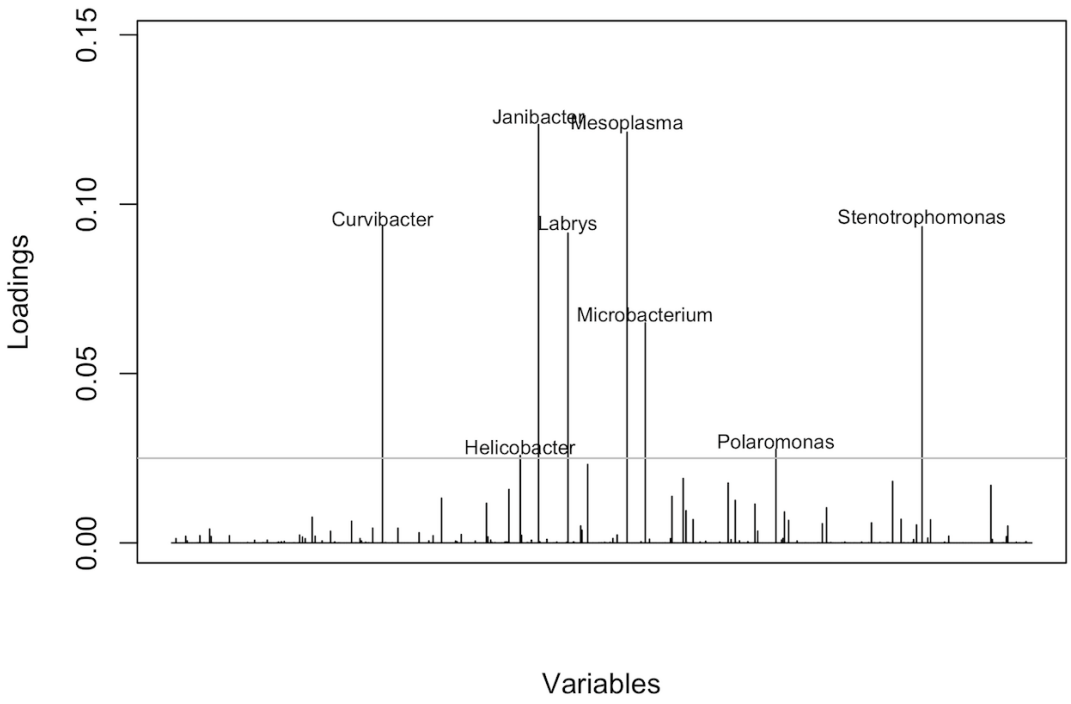


A



B

Supplementary figure 2. First and second principal components coloured by **(A)** age group and **(B)** gender. Spots indicate individuals. Red arrows indicate loadings.



Supplementary figure 3. Relative contributions of genera driving difference between active trachoma cases and controls.

CHAPTER 7: NOTES AND ADDITIONAL INFORMATION

7.3 Note A: Comparison of extraction technique for organism detection by PCR

7.3.1 Introduction

A critical step in detection of pathogen DNA with NAATs is release of the genomic material from bacterial bodies. One weakness of PCR diagnostics for bacteria is that the differing chemical composition of bacterial cells walls make them variably resistant to chemical lysis techniques. Lysis procedure has been shown to influence the evenness of species recovery in microbiota studies (316). This issue can be compounded by the complexity of the specimen, which may further inhibit DNA release, although this is unlikely to be the case for low-biomass conjunctival specimens. A DNA and RNA co-purification protocol was used in this study in order to provide the option for future experiments on host gene expression. RNA is highly susceptible to degradation and, as a result, the lysis technique does not involve harsh lysis steps such as bead-beating or prolonged incubation at elevated temperatures. Cell wall disruption is achieved with proprietary detergent-based lysis buffer. *Ct* has been detected at high loads in swabs extracted using RNA-tolerant lysis buffers in this and other previous studies (74,312,313), however, publications using this particular lysis technique followed by detection of other common pathogens are lacking. I therefore set out to compare nucleic acid recovery from key bacteria from our technique compared to another commonly used protocol with more severe lysis methodology to assess whether it may have influenced diagnostic performance.

7.3.2 Methods

7.3.2.1 Preparation of bacterial isolates

Cultured isolates of *Sp, Hi, Mc, Staphylococcus epidermidis* (representing CoNS, chosen as the most commonly found CoNS on the skin and eyes) and *Sa* were grown from laboratory stocks using standard microbiological techniques.

Lysis of adenovirus particles was not assessed during this study because viral DNA is readily available in the cytoplasm of host cells during an infection, and the lysis of human cells with this technique was already confirmed by detection of the *H. sapiens* RPP30 endogenous control target.

7.3.2.2 Nucleic acid extraction and detection

Each of the antecedent studies from which these assays were taken utilized different extraction protocols for isolation of DNA from cultured colonies and clinical specimens. The techniques are outlined in Table 7.3.2.2.1. The QIAamp DNA mini kit was selected as a comparator kit because

it was commonly used, similar in protocol to the spin column kits used in many of these studies, and included enzymatic digestion of material for lysis prior to heating with proteinase K.

Table 7.3.2.2.1. Lysis and extraction techniques used during qPCR assay validation.

Test	Specimen type	Extraction kit	Lysis	Ref
H. influenzae fucK	Cultured reference strains, blood, CSF	Gentra PureGene Extraction kit	Not specified – manufacturer recommends incubation in proprietary Cell Lysis Solution at 80°C for 5 mins.	(314)
M. catarrhalis copB	Cultured reference strains, nasopharyngeal swabs	QIAamp tissue kit	Lysed using 0.5% SDS, proteinase K, heated to 55°C for 1 hour.	(315)
S. pneumoniae lytA	Cultured reference strains, serum, middle ear fluid, CSF.	QIAamp DNA kit	Triton X100/mutolysin/lysozyme-supplemented lysis buffer heated to 37°C for 1 hour	(316)
S. aureus SpA	Cultured references strains, lab-confirmed staph positive blood cultures.	Custom	Colonies resuspended in water and heated at 98°C for 15 minutes and then centrifuged to remove bacterial debris	(317)
Coagulase-negative *Staphyloccocus tuf*				

CSF: cerebrospinal fluid; SDS: sodium dodecyl sulfate (detergent).

Approximately 20µL of bacterial cultures were scraped from culture plates and resuspended in 1X PBS. Four ten-fold dilutions of resuspended bacterial cultures were prepared and each was aliquotted into six separate 200-µL aliquots. These aliquots were randomly allocated to Qiagen AllPrep DNA/RNA kit (Qiagen, Manchester, UK) extraction and Qiagen QIAamp DNA mini kit (Qiagen, Manchester, UK) extraction with manufacturers recommendations for lysis.

The lysis procedure for the Qiagen AllPrep DNA/RNA kit involved vortexing for one minute in proprietary RLT Plus buffer (Qiagen, Manchester, UK) supplemented with β-mercaptoethanol followed by incubation for five minutes at room temperature. Lysis for the Qiagen QIAamp DNA mini kit involved lysis with 10mg/mL lysozyme (Sigma Aldrich, Gillingham, UK) for 20 minutes at 37°C followed by 60 minutes incubation at 56°C with proprietary buffer AL (Qiagen, Manchester, UK) supplemented with proteinase K.

For both procedures, the lysed material was subsequently bound to the column, washed and eluted into 100µL of 0.1✕ Tris EDTA as per respective recommendations for each kit.

Equal volumes of eluate were tested with ddPCR as described in the manuscript in section 7.2.

7.3.3 Results

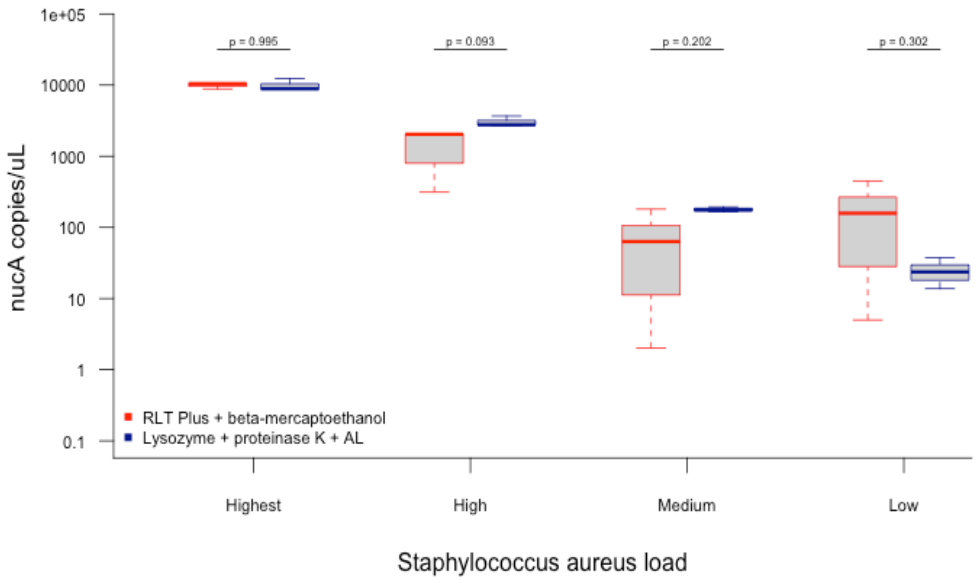
Figure 7.3.3.1 illustrates the difference in yield recovered from bacterial isolates lysed using differing lysis methods. Both lysis procedures lysed all bacterial suspensions effectively regardless of input concentration. For *Mc*, *Sp* and *Hi*, the AllPrep DNA/RNA kit consistently

offered improved recovery of genomic targets as compared to the QIAamp DNA kit with the exception of the highest concentration *Hi* where recovery was similar. For *Sa* and *S. epidermidis*, there were consistently fewer targets recovered in those samples extracted using the AllPrep DNA/RNA kit than from those extracted using the QIAamp DNA kit at high and medium load input. At highest input load, both kits yielded the same number of target copies for both *Sa* and *S. epidermidis*. However, across all loads, the mean reduction in *S. epidermidis* load was 47.7%, and the mean reduction in *Sa* load was 33%.

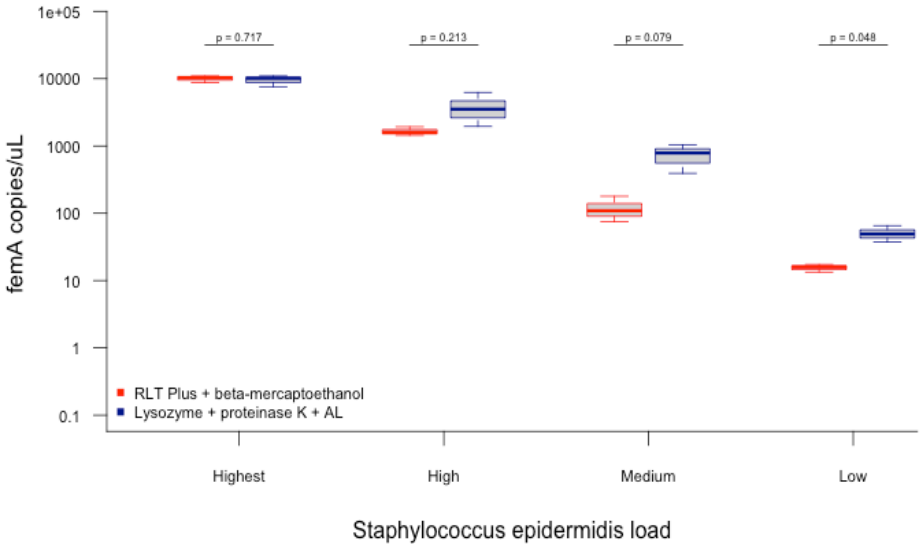
7.3.4 Discussion

It is possible the loss of material from the incomplete lysis may impact on targeted staphylococcal assay sensitivity. During validation of the qPCR protocol from which these oligonucleotide sequences were taken, the study authors purified DNA by resuspending colonies in water and heating at 98°C for 15 minutes and then centrifuging them to remove bacterial debris, a relatively crude technique which still resulted in successful release of genetic material for PCR (317). Adding mechanical lysis to chemical/enzymatic lysis is recommended for optimal evenness of representation of phyla, however, it is known to impact DNA yield (318). The prevalence of some difficult-to-lyse bacteria may have been under-represented on both the targeted testing and the community profiling. However, the lysis method was consistent between cases and controls therefore comparison between those two groups is still appropriate. As the lysis technique does not result in complete loss of any of the ddPCR targets, the assays would still be able to detect high-load targets, which are those most likely to be causing inflammation.

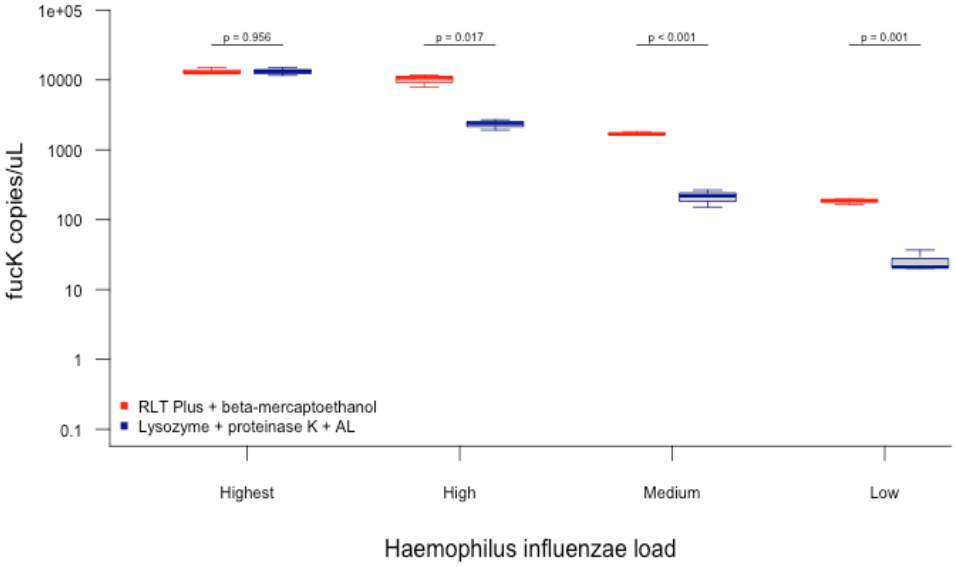
A


1e+05
10000
1000
100
10
1
0.1
nucA copies/uL
p = 0.995
p = 0.093
p = 0.202
p = 0.302
RLT Plus + beta-mercaptoethanol
Lysozyme + proteinase K + AL
Highest
High
Medium
Low
Staphylococcus aureus load

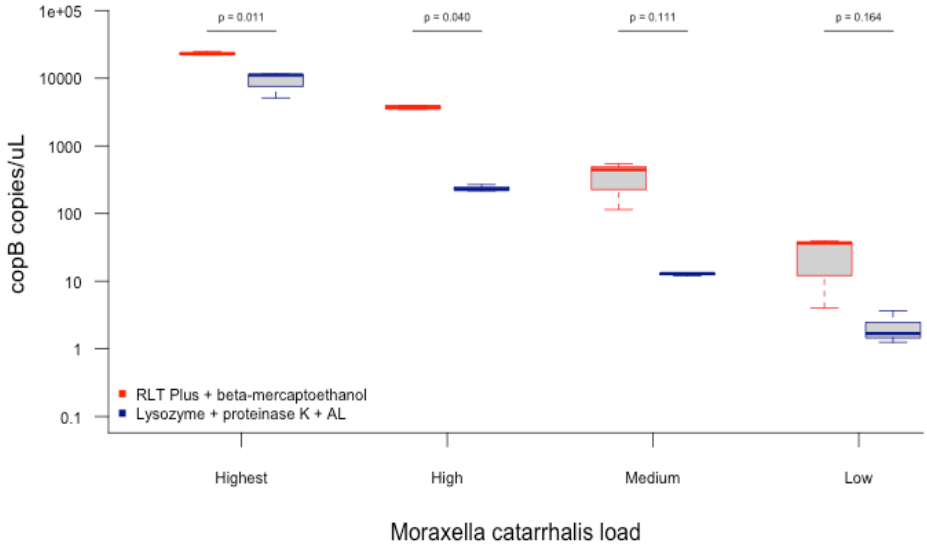
B


1e+05
10000
1000
100
10
1
0.1
femA copies/uL
p = 0.717
p = 0.213
p = 0.079
p = 0.048
RLT Plus + beta-mercaptoethanol
Lysozyme + proteinase K + AL
Highest
High
Medium
Low
Staphylococcus epidermidis load

C


1e+05
10000
1000
100
10
1
0.1
fucK copies/uL
p = 0.956
p = 0.017
p < 0.001
p = 0.001
RLT Plus + beta-mercaptoethanol
Lysozyme + proteinase K + AL
Highest
High
Medium
Low
Haemophilus influenzae load

D


1e+05
10000
1000
100
10
1
0.1
copB copies/uL
p = 0.011
p = 0.040
p = 0.111
p = 0.164
RLT Plus + beta-mercaptoethanol
Lysozyme + proteinase K + AL
Highest
High
Medium
Low
Moraxella catarrhalis load

E

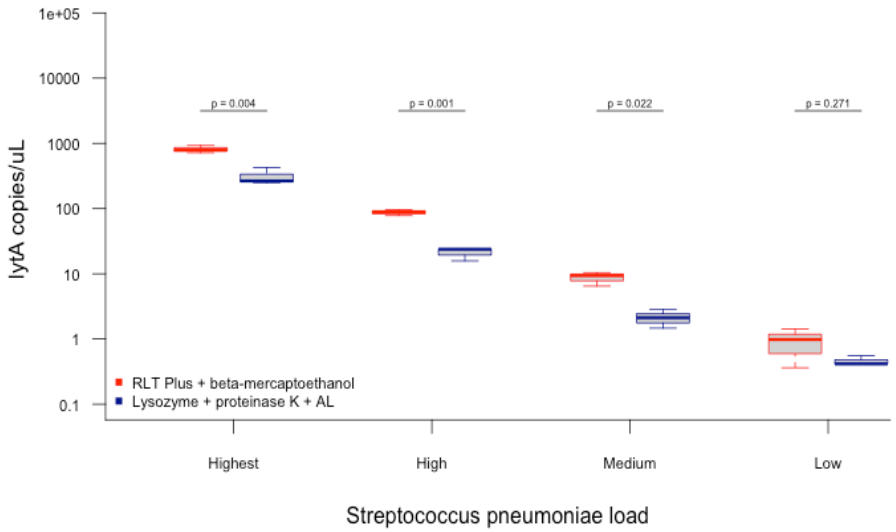


Figure 7.3.3.1. Comparative load recovered from bacteria targeted in this study. (A) *Staphylococcus aureus*, **(B)** *Haemophilus influenzae,* **(C)** *Staphylococcus epidermidis*, **(D)** *Moraxella catarrhalis* and **(E)** *Streptococcus pneumoniae* when extracted using Qiagen AllPrep Mini DNA/RNA kit (red boxes) and Qiagen QIAamp DNA mini kit (blue boxes). Boxes represent mean and interquartile range, and whiskers represent 1.5-times the interquartile range. RLT Plus and AL are proprietary lysis buffers.

7.4 Note B: Agreement between droplet digital PCR and 16S gene sequence representation

7.4.1 Introduction

Both 16S amplicon sequencing and ddPCR utilize thermocycling and DNA polymerase to amplify gene sequences to detect bacteria. ddPCR specifically targets conserved gene regions which should make the reaction more efficient and predictable, whereas even the conserved regions of the 16S gene where primers bind do have some diversity. Additionally, the V-regions within the 16S amplicon are highly variable in terms of length and sequence, and therefore the efficiency of amplification will not be equal across all species. The individual characteristics of each process is appropriate for their purpose in this study; ddPCR must be specific as a diagnostic, whereas it is necessary for 16S amplicon sequencing to misrepresent some peripheral genera because it profiles the community composition as a whole.

Both techniques were applied to the subset of specimens from which 16S gene sequences were amplified, thereby allowing comparison between the two. ddPCR was used to test for bacterial species in four different genera: *Staphylococcus, Streptococcus, Moraxella* and *Haemophilus*. *Staphylococcus* and *Streptococcus* were excluded from the primary analysis of clinical specimens as they were found in the 16S negative controls. Neither *Haemophilus* nor *Moraxella* had significant influence on the first, second or third principle components identified in the dataset. None of the bacteria tested with ddPCR were significantly associated to clinical status.

7.4.2 Methods

16S amplicon sequencing and ddPCR was conducted as described in the manuscript in section 7.2.

16S sequence read number was compared to ddPCR result. For the purposes of the comparison, the ddPCR result was considered the ‘true’ result. Diagnostic comparison was conducted using varyingly stringent diagnostic criteria. Two ddPCR diagnostic criteria were tested: any load above 0 copies/µL (including those where the load is so low our confidence in non-zero estimate is below 95%; less stringent), and any ddPCR positive result (where only those with sufficient load to give us over 95% confidence in non-zero load; more stringent). These diagnostic criteria have been outlined previously (231). Three thresholds for classification of 16S amplicon positivity were set (>0 [least stringent], >10, >100 [most stringent]). Subsequently, Fleiss’ unweighted kappa was calculated to determine agreement between specimens. Chi-squared tests were employed to determining whether there was a significant association between ddPCR positivity and 16S read number at each level of stringency.

The number of genus-specific reads were compared in all ddPCR negative versus all ddPCR positive specimens. Logistic regression was used to test whether there was a relationship between ddPCR positivity and 16S read number.

7.4.3 Results

Table 7.4.3.1 provides a summary of diagnostic comparisons between the two techniques. None of the results show a significant association between number of genus-specific 16S reads and ddPCR result. For all four genera, there was a large number of specimens with 16S reads for each genera but ddPCR negative (16S>0/ddPCR- *Staphylococcus*: 85; *Streptococcus: 85; Moraxella*: 28; *Haemophilus*: 59). This was reflected by very poor specificity and PPVs. The overall diagnostic potential of 16S sequencing is very poor Kappa agreement indicated mostly ddPCR and 16S sequence presence had no better than random agreement. Some (albeit poor) agreement was reached at the most stringent diagnostic criteria (ddPCR 95% confidence in non-zero load, >100 genera-specific 16S sequences), suggesting the need for cautious quality control with this technique. However, even at the most stringent level, diagnostic performance was far below the level normally required for use. This was true across all four genera.

Box plots comparing number of 16S reads in all ddPCR-negative (at the 95% confidence level) specimens to all ddPCR-positive specimens tested. In general, the number of reads was higher in ddPCR-positive specimens, but this was hugely variable, and the group sizes were too small for meaningful analysis. Many of the ddPCR-negative specimens which detected a target load below the threshold for positivity also had 16S reads detected at variable levels. There were several ddPCR+/16S- specimens in the (*Hi fucK*: 3; *Mc copB*: 1), although the vast mojrity of discrepant results were ddPCR-/16S+.

7.4.4 Discussion

There was no agreement between ddPCR and 16S. This was true regardless of how stringent the diagnostic criteria. There was also no association between load detected by PCR and number of 16S sequences matching that genus. This finding is surprising, however, it does not detract from the overall message of the manuscript presented in section 7.2 which uses these two techniques to address completely different questions. ddPCR was used as a sensitive and specific diagnostic in the absence of available culture facilities, and 16S was used to characterise the overall structure of microbial communities at the conjunctiva.

The ddPCR target for three of the genera (*Streptococcus*, *Moraxella* and *Haemophilus*) are species-specific, yet other species in those genera are also common components of the skin microbiota. These other species may explain the number of reads in some of the 16S+/ddPCR- specimens. For example, viridans-group *Streptococci* and non-*influenzae Haemophilus* species have frequently been cultured from the conjunctivae. Many earlier culture-based studies do not

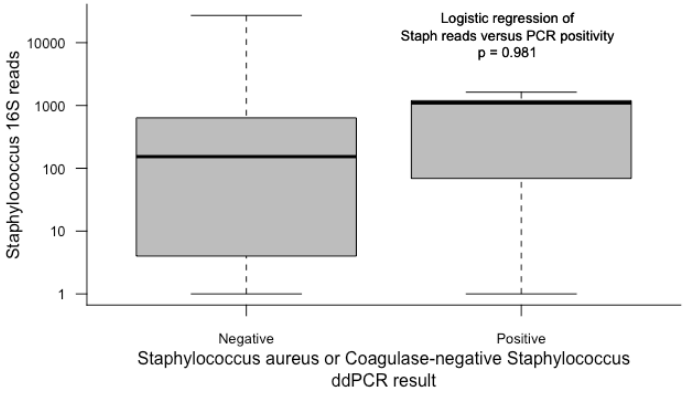
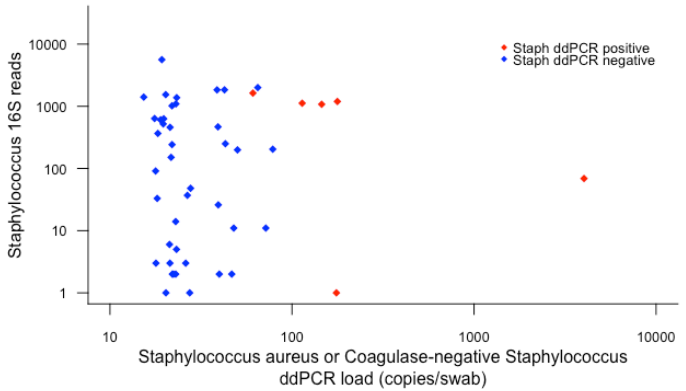
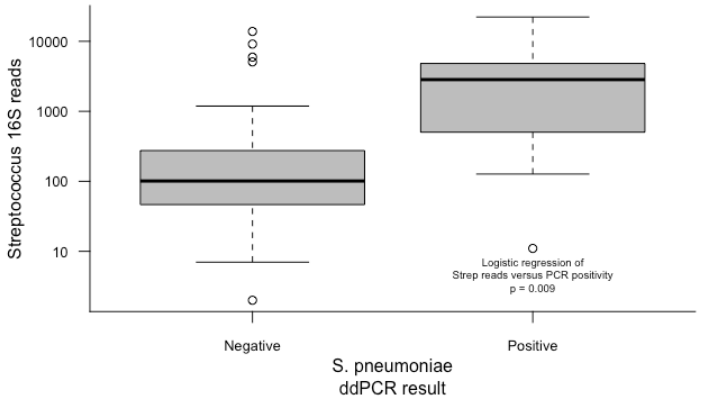
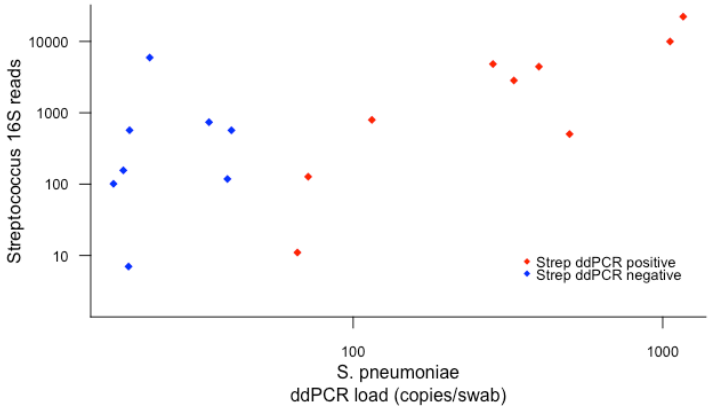
go into species-specific differentiation of these groups, and there is likely to be some commensal bacteria within the genera which generate genera-specific 16S sequences, but do not amplify with species-specific primers. However, for *Staphylococcus*, we specifically used broad spectrum primers which detect a number of different *Staphylococcus* species in the coagulase-negative group (317). These are frequently classed among the most prevalent members of the normal ocular flora (319). Several specimens were identified where a positive load of *Staphylococcus* was recorded, but the diagnostic result was deemed negative because the concentration was too low to be classified as a positive PCR result. This may be an artifact of the extraction process, as described in the manuscript and in Note A. This is especially relevant as the prevalence of CoNS was much lower than might have been expected in this group. Alternatively, staphylococcal contamination was present in the no-template controls, suggesting it might also be an artifact of the sequencing process. When the criteria for classification as a positive result are relaxed, 16S sequence number becomes more specific for ddPCR result. However, this is reflected in a compromise in sensitivity. Other aspects of the assay therefore seem likely to be causing multiple false positive 16S results, perhaps related to species not targeted by the PCR assays. Optimisation of the 16S library preparation and bioinformatics pipelines may improve correlation between sequence data and targeted PCR, which would enable us to study the constituents of these communities with more accuracy.

Table 7.4.3.1. Diagnostic correlation between 16S reads and droplet digital PCR positivity. *In all of the diagnostic analyses, ddPCR was treated as the 'true' result and 16S read number as the comparator. Using Bonferroni's correction for multiple testing, a p-value of 0.002 was considered significant.*

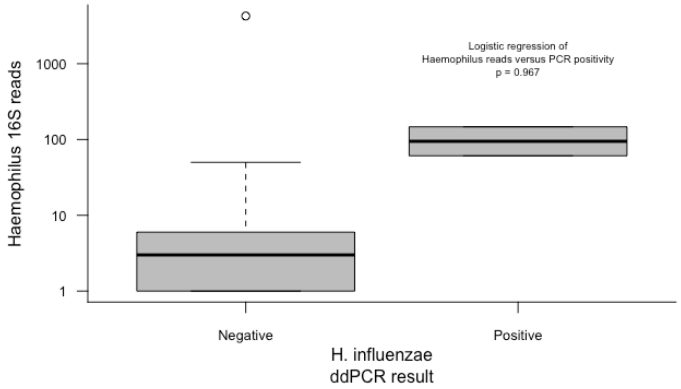
Genera	ddPCR	16S reads	Sensitivity	Specificity	PPV	NPV	Cohens Kappa	Chi-squared p-value
Staphylococcus	Load >0 copies/µL	>0	0.940	0.043	0.516	0.400	-0.017	1.000
		>10	0.739	0.140	0.315	0.429	-0.060	0.680
		>100	0.500	0.391	0.472	0.419	-0.109	0.387
	Positive	>0	1.000	0.056	0.066	1.000	0.007	1.000
		>10	0.833	0.300	0.074	0.964	0.023	0.817
		>100	0.667	0.456	0.075	0.953	0.026	0.874
Streptococcus	Load >0 copies/µL	>0	1.000	0.025	0.181	1.000	0.009	1.000
		>10	0.762	0.063	0.178	0.833	0.002	1.000
		>100	0.882	0.532	0.288	0.955	0.229	0.005
	Positive	>0	1.000	0.023	0.096	1.000	0.004	1.000
		>10	1.000	0.069	0.100	1.000	0.014	0.928
		>100	0.889	0.494	0.154	0.977	0.122	0.065
Haemophilus	Load >0 copies/µL	>0	0.706	0.380	0.197	0.857	0.043	0.698
		>10	0.039	0.937	0.375	0.841	0.143	0.295
		>100	0.059	0.987	0.500	0.830	0.071	0.785
	Positive	>0	0.400	0.352	0.033	0.914	-0.040	0.518
		>10	0.400	0.934	0.250	0.966	0.260	0.072
		>100	0.200	0.989	0.500	0.957	0.264	0.203
Moraxella	Load >0 copies/µL	>0	0.167	0.700	0.036	0.926	-0.049	0.817
		>10	0.000	0.989	0.000	0.937	-0.011	1.000
		>100	0.000	0.989	0.000	0.937	-0.011	1.000
	Positive	>0	0.000	0.705	0.000	0.985	-0.021	1.000
		>10	0.000	0.989	0.000	0.989	-0.011	1.000
		>100	0.000	0.989	0.000	0.989	-0.011	1.000

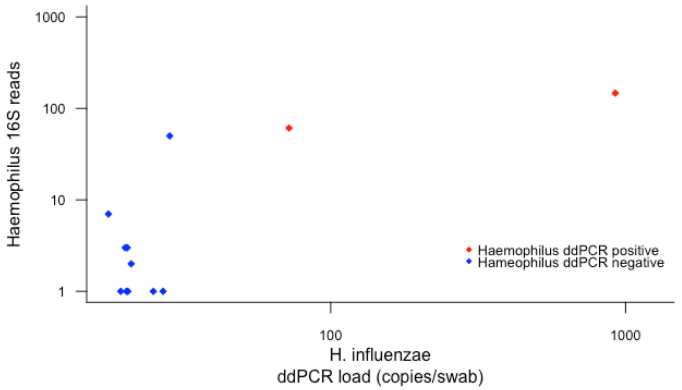
ddPCR: droplet digital PCR; PPV: positive predictive value; NPV: negative predictive value.

Table 7.4.3.2. Graphical comparison data between droplet digital PCR and 16S gene sequence reads. Boxes in column two represent interquartile range and whiskers depict 1.5-times the inter-quartile range

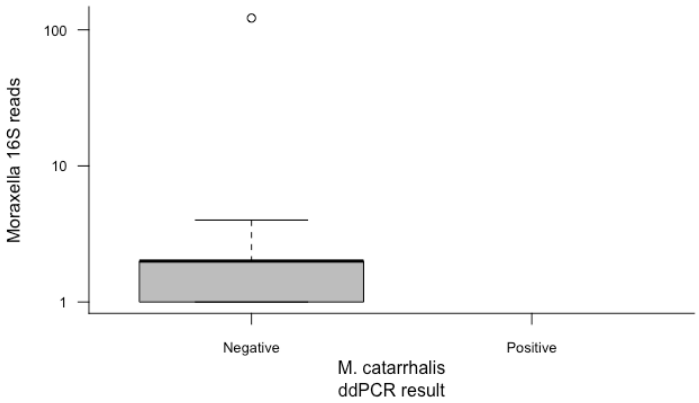
Organism	Number of reads versus PCR result	Number of reads versus PCR load
Staphylococcus		
Streptococcus		

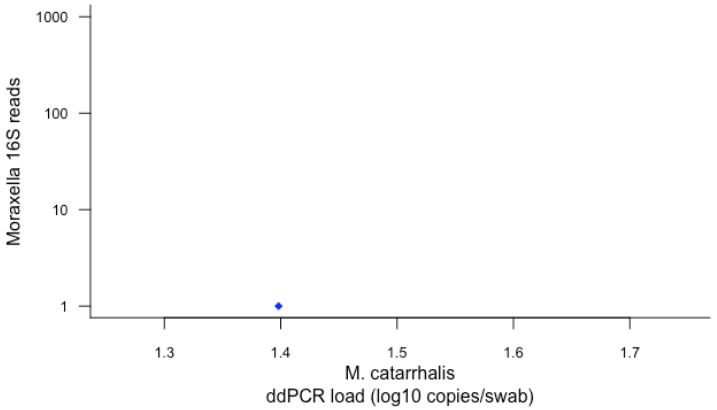
Haemophilus





Moraxella





8. DISCUSSION

Evidence on the sensitivity and specificity of TF for *Ct* infection strongly suggests that after MDA, or in low prevalence environments, the PPV of TF is low (126). In this thesis, we present data suggesting in the Solomon Islands the PPV is also low, even though the level of TF is moderately high, and no treatment has yet been administered. This might be attributable to the unmasking of phenotypically similar diseases as the global prevalence of ocular *Ct* infection drops and as an increasing number of people from different parts of the world are examined. Research into the appropriateness of diagnostic tools for monitoring and evaluating public health programs is therefore critical. Interventions for trachoma were developed largely based on data generated in sub-Saharan Africa, and the appropriateness of tests that work well in endemic African countries should be explored in other world populations. The study of trachoma in the Pacific is therefore justified, and the outcomes have important implications locally and for other countries considering elimination.

8.1 Data synthesis

The first hypothesis for this study was that TF meeting the criteria for intervention is not concurrent with sufficiently prevalent TT to be classed as a public health threat; we suspect the clinical phenotype in the Pacific Island small states to be unusual. Vanuatu and Solomon Islands were shown to have TF present at levels that require A, F and E intervention, but TT prevalence is too rare to be counted as a public health threat according to WHO thresholds. In rejecting the null for the first hypothesis of this study, we find a paradox in Melanesia – the TT prevalence suggests trachoma is not a public health problem in Solomon Islands, Vanuatu and Fiji, but treatment is indicated based on the prevalence of TF. TF is not sight-threatening in its own right, nor even symptomatic in most cases. It is also possible that this is isolated to the region. However, the presentation of trachoma in endemic populations is likely to be diverse as a result of the life-long accumulation of stimuli required to develop scarring and TT, therefore the relationship between TT and TF is not expected to be linear. In figure 1.3.2, there are a small number of studies where a similar pattern is observed (TF ≥10% in those from 1–9 years, TT < 0.2% in those aged 15+ years). However, these estimates were not, for the most part, generated using standardised study design and grading, nor were they age standardised in most cases. They also included single districts in studies where many of the other districts did have a public health threat from TT (for example, Cameroon (52)). The GTMP is expected to identify further districts where the work presented in this thesis may be relevant; infection testing may be warranted in those areas. Elsewhere in the Pacific, more cases of TT were found in one survey in Kiribati than the other three surveys in Solomon Islands, Vanuatu and Fiji combined. Kiribati met the WHO criteria for intervention, and should be reviewed for capacity to implement the S component of the SAFE strategy immediately in addition to implementation of A, F and E components of trachoma control. These are a multitude of differences between Melanesia and Micronesia that might lead to differing trachoma endemicity; this may be an area of interest for future studies but beyond the scope of this study.

The second hypothesis of this study was that in these areas where TT is rare, there was no association between ocular *Ct* and TF. The population prevalence of TF and *Ct* are well correlated in many endemic districts, but situations are emerging (e.g., low prevalence, post-treatment) where TF is not closely associated with *Ct*. As an increasing number of districts embark on control programmes and others consider whether elimination goals have been met, the diagnostic specificity of trachoma for *Ct* infection should come under increased scrutiny. Prior to this study, the prevalence of TF and TT was not clear in Fiji; recent reports had conflicting evidence. More recent studies have indicated the high TT prevalence estimate to be an artefact of the WHO simplified system and social practices of eyelash epilation among Fijians (304), and suggest the prevalence in the Western Division to be 0%. We have now demonstrated ocular *Ct* infection to be scarce and not associated with TF, thereby addressing the second hypothesis in Fiji. Subsequently, the seroprevalence of anti-Pgp3 antibodies was found to be low in Fiji and did not increase with age (305). The second study hypothesis was further investigated in the Solomon Islands. *Ct* was associated with TF in this population; however, TF was observed in more than a quarter of children surveyed, yet over 95% of them did not have *Ct* infection. Independent, long-lived antibody markers of *Ct* infection are less susceptible to artefacts from seasonal or temporal fluctuations in transient infection - these did not suggest accumulation of *Ct* exposure. The clinical signs of progressive conjunctival fibrosis were not prevalent in adults, suggesting the trachoma was not progressing as severely as it does elsewhere. Therefore, in the Solomon Islands, several indicators (TI, severe scars, *Ct* infection, anti-Pgp3 antibodies and TT) are practically absent, yet TF is still present at levels that warrant intervention. The implications for Melanesia have been that two rounds of MDA have been reconsidered, giving Ministries the opportunity to invest resources and staff elsewhere. The cost of MDA in the Pacific Islands is known to be high, in part due to their wide geographic distribution, and this could represent a significant saving.

The third study hypothesis was that an alternative pathogen may be more closely associated with TF than *Ct.* The evidence did not suggest that the prevalence of nonchlamydial pathogens was disproportionately higher in the eyes of those with TF compared to those without, therefore the null hypothesis could not be rejected. Non-*Ct* infections are thought to contribute towards inflammation that causes scarring to progress (11,39), therefore this finding is consistent with the low prevalence of severe scars in the population. Upon examination of the full bacterial community at the conjunctiva no shift in the richness or diversity of genera represented was observed when comparing cases of TF with matched counterparts, suggesting bacterial dysbiosis is not associated with TF here, and therefore unlikely to be the cause of active disease in people with no serological evidence of *Ct* exposure. Together, the data suggest there is a low prevalence of trachoma in the Solomon Islands that is attributable to *Ct* infection. This accounts for the low prevalence of severe scars and TT cases. It is possible the burden of TF is being inflated by a viral or allergic cause, although the epidemiology and presentation of those types of conjunctivitis are well characterised and distinct from those of trachoma. Figure 8.1.1

presents a summary of the data presented in this paper, and a proposed model for trachoma progression in the Solomon Islands resulting in low prevalence of TT.

The underlying reasons for the proposed difference in pathogenesis are hard to speculate. The genetics of the pathogen are broadly similar to other serovars that cause trachoma. Even at those loci thought to have disproportionate influence on virulence (mostly found in the PZ), the Solomon Island strains are similar to other ocular strains. Cases of TF in the absence of detectable *Ct* is not unprecedented; studies have found the PPV of TF for NAAT positivity to be low in areas where TF prevalence exceeds 10% (31), but this is not commonly observed in a treatment-naïve population. Has this community previously been exposed to antibiotics? There is no suggestion of mass macrolide distribution or increased availability compared to other trachoma-endemic environments in the academic literature. Trachoma is typically associated with arid areas where water is so limited that it restricts face washing behavior. Fresh water is commonly available in the Solomon Islands due to the regular, year-round rainfall (figure 1.9.5). Therefore, the environment is clearly different to many (although not all) trachoma-endemic countries, yet 60-70% of children in some provinces have visible oculonasal secretions upon examination ('dirty face') (287). Greater distance to nearest source of water for washing or drinking was not associated with trachoma in the Solomon Islands during the GTMP survey in 2013 (290). According to recent surveys, approximately 60% of households in Solomon Islands do not have access to improved latrines, suggesting availability of fly breeding sites near houses, but there was no association between absence of latrines and trachoma (290). It is possible our surveys have taken place in at a seasonal nadir of infection transmission, however, the consistency of the weather depicted in figure 1.9.5 suggests the Solomon Islands do not experience seasonality in rainfall and temperature, and therefore trachoma may also not be subject to profound seasonal variation on that basis. Host genetics may have a role to play as the population of the Solomon Islands is genetically isolated, but as mentioned in the discussion of the manuscript in section 5.2, some of the alleles that have associated with increased risk of scarring are present at high frequency throughout the Pacific. Urogenital infections are also highly prevalent, suggesting they are not impeded by local host resistance to *Ct* infection.

The profile of age-specific TF prevalence in the pre-MDA survey could suggest an alternative transmission pattern. It is possible that ocular *Ct* infections are being transmitted but do not establish infections of sufficient duration or intensity to drive antibody responses and immunopathology. It is tempting to consider whether the high prevalence urogenital *Ct* infections in expectant mothers may have an impact on susceptibility to *Ct* infection, or the inflammatory response to a single episode of *Ct* exposure. The prevalence of urogenital *Ct* infections in Melanesia is among the highest in the world. In chapter 6, this is suggested as a potential reason for the high proportion of 1-year olds in this study with antibodies to Pgp3. While antibodies alone to *Ct* are insufficient to provide protection against infection, sustained antibody levels throughout childhood may be indicative of a broader immune response that may give children in these communities an immunological 'head start' against ocular *Ct* infection.

There may also be a herd effect of high proportion of seropositive young children in the community acting as 'barriers' to widespread transmission, perhaps even a critical mass of seropositive individuals within a population. There are critical limitations to this hypothesis; firstly, any relationship between exposure *in partum* and either protection against subsequent infection or attenuation of response to single infection stimuli would be expected to show some association (either negative or positive) with high anti-Pgp3 antibody titre. This is not the case in this population. Secondly, the formation of lymphoid centres to facilitate B-cell maturation, class switching to IgG and plasma cell formation may not be possible at such a young age with such transient exposure. Thirdly, in endemic settings, multiple infections are thought to be needed to induce scarring, and the accumulation of exposure enhances risk of scarring. Therefore, the concept of early exposure attenuating response to future challenge seems counterintuitive. Ultimately, the current understanding of the basic biology of protective immunity to *Ct* is insufficient to speculate whether this is plausible. Further research into the antibody status of children born to mothers with active urogenital infection, longitudinal short- and long-term follow-up might provide better understanding of how this affects an individual's response to subsequent *Ct* stimuli.

8.2 Limitations and future opportunities

The islands in this study are separated by hundreds of kilometers of open ocean, and the majority of the populations are rural and isolated. The geographical isolation promotes microclimates throughout the archipelago, therefore the likelihood of disease being homogenous throughout the region is low. This is demonstrated by the difference observed between Temotu and Rennell & Bellona, political neighbors with differing dominant ethnicities, very little migration between the two, and apparently differing levels of trachoma and infection. The effect of MDA is also not apparently consistent between the two provinces; surprisingly, in villages in Rennell & Bellona where infection and TF were more closely correlated, the impact of MDA was negligible, whereas in Temotu where over 95% of TF cases do not have detectable *Ct* infection, TF was reduced by over 50%. Preliminary estimates of MDA coverage were approximately 70% in Rennell & Bellona and 90% in Temotu. The study was not powered to detect differences between provinces, and the number of villages is so low, it is likely to be susceptible to inaccuracies due to disease clustering. It is also not surprising that a broad spectrum antibiotic with immunomodulatory effects should reduce an inflammatory phenotype such as TF even in the absence of a detectable macrolide susceptible causative agent. However, this does suggest that, as long as suitable coverage is achieved, MDA is likely to be effective at reducing TF in this population. While cases of TT may be rare in Temotu, six cases were found in the Western province of the Solomon Islands during the GTMP, and others have been referred to central clinics from Malaita province (figure 1.9.3). There is likely to be substantial geographical variation in trachoma prevalence, therefore data collected from surveys of one island group should be applied cautiously to other areas. Further work is warranted to extend the geographic coverage of the Pacific trachoma programme, and support

national programmes in directing their trachoma elimination efforts to pockets of trachoma which may remain on some islands.

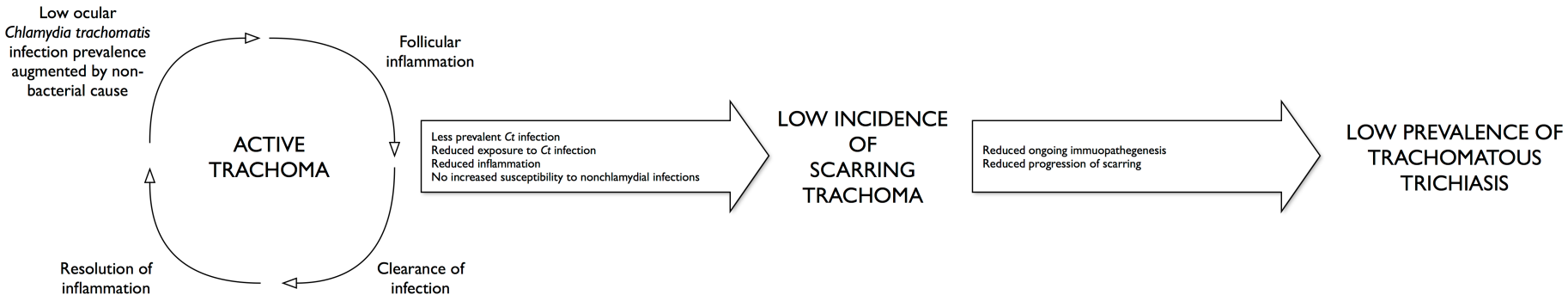


Figure 8.1.1. Proposed mechanism of trachoma pathogenesis in the Solomon Islands.

It remains unclear whether nonchlamydial pathogens are an independent causative agent for TF or whether they are opportunistic pathogens of conjunctivae that have previously been conditioned to be more susceptible to repeated *Ct* infection. The data in section 7.2 support the latter hypothesis, but the TF in the absence of antibodies to Pgp3 support the former. No relationship was found between clinical signs and pathogens commonly isolated from trachoma-endemic settings, yet the ocular *Ct* endemicity of study populations where the majority of data have been generated may introduce bias that precludes comparison to the Solomon Islands. Because TF is present in Melanesia children and declines into adulthood, a single cause seems more likely, as acquired partial immunity to a mixture of causes may develop more slowly. However, in the absence of a definitive pathogen associated with follicular inflammation, there is no 'smoking gun' upon which to form further hypotheses about a potential different cause. Microarray or sensitive amplification-free deep sequencing technologies could shed light on a potential causative agent, and longitudinal investigations would be warranted to determine causality.

In trachoma-endemic environments, the vast majority conjunctival scarring is considered to be a result of *Ct* infection. However, nonchlamydial and noninfectious causes of scarring are intermittently mentioned in the literature (320,321). Although many adults with scarring are also seropositive for anti-Pgp3 antibodies, we have presumed that most of the anti-Pgp3 signal is those adults is caused by previous urogenital infection. The accumulation of minor scars with increasing age in seronegative individuals suggests there may be other causes of incident scarring that do not progress to scarring severe enough to distort an eyelid, but without a longitudinal study of scarring, it is not possible to definitively declare the risk of progression. Although no trachoma intervention is directly aimed at treating scarring, it is the primary cause of entropion and therefore improving our understanding of the causes and mechanisms of scarring is critical. In the peri-elimination period, TS may prove a useful short-term indicator of whether A, F and E interventions are likely to lead to reduced risk of developing TT (34,100). There are also likely to be parallels between eyelid scarring and pelvic organ scarring (a complication of vaginal *Ct* infection that can lead to infertility and ectopic pregnancy), therefore improved understanding of scarring pathogenesis could help us identify what aspects of *Ct* infection are critical to inducing ongoing immunopathogenesis.

In these communities, TF appeared to be successfully treated by MDA where coverage was sufficient. A final issue for consideration is whether the communities would benefit from trachoma-targeted interventions for reasons other than trachoma control. There has already been a positive impact of the first MDA round on yaws (102) and sexually transmitted infections (322); other beneficial impacts of MDA are described in section 1.5. In Fiji, yaws does not appear to be endemic (305), so the collateral benefit of azithromycin MDA may be limited in that respect. The potential benefit of F and E implementation in parts of Melanesia is significant, where many still live traditional subsistence lifestyles and there is social stigma associated with insufficient infrastructure for basic hygiene practices. The negative implications of MDA should also be considered. Some commentators have suggested that disrupting the natural

accumulation of partial immunity within the community could theoretically have negative knock-on effects to the prevalence of *Ct,* however, this is yet to be empirically proven (114). The most pressing risk from MDA is the emergence of macrolide-resistant pathogens. Resistance to macrolide antibiotics among, for example, *Mycoplasma genitalium* (323), *Treponema pallidum* (324) and *Neisseria gonorrhoeae* (325) isolates is emerging worldwide at an alarming rate (326). Current published data from trachoma studies has not demonstrated significant drop in antibiotic susceptibility among most pathogens studied. Where macrolide-resistance has been observed, it seems to decrease after the removal of antibiotic pressure (113). However, prospective, long-term studies in communities where MDA is undertaken for a prolonged period are lacking. These would be especially valuable in communities where more intensive regimens (e.g., biannual treatment) are under consideration.

On balance, this candidate feels that the momentum of the trachoma community could be effectively leveraged to provide holistic benefit to the lives of Pacific Islanders, particularly from the F and E components of SAFE strategy. In the absence of compelling evidence that trachoma MDA specifically drives resistance, this candidate feels this should include the A component, pending urgent studies on the potential negative implications of MDA. No doubt those struggling to eliminate trachoma with already over-stretched budgets may disagree.

8.3 Conclusions

In the Solomon Islands, the prevalence of TF was moderate before MDA, but was poorly predictive of *Ct* infection positivity. TF was reduced by approximately 50% after MDA, yet ocular *Ct* prevalence did not change. TF was not associated with exposure to Pgp3, and adults in the same population did not have a high prevalence of severe scarring, nor sufficient TT to be classed as a public health problem. Does TF therefore represent the burden of ocular *Ct* endemicity in this area, or the need for trachoma intervention? The evidence suggests not. International guidelines recommend basing decisions about the implementation of A, F and E on TF alone; in this context, that guideline has led to significant spending on interventions aimed at treating an infection that does not appear to be prevalent.

To the credit of the GET2020 community, MHMSs in the Pacific and international partners have embraced the findings of this study and adopted a pragmatic approach to trachoma elimination. The Pacific Trachoma Initiative, assembled in response to these data, has curtailed the MDA programme pending more data and has integrated pre-, short-term (6 months) post- and long-term (24 months) post-MDA operational surveillance of ocular *Ct* infection into the Trachoma Action Plans of Solomon Islands, Vanuatu, Fiji and Kiribati. This will provide additional data to Ministries to inform their decisions about whether additional rounds of MDA are warranted after the first is complete.

As many as eight annual MDA treatments have incompletely controlled TF in some settings in Africa and this may be an indication that the situation in the Pacific region is not unique. Whilst the 'unusual' clinical presentation that triggered this study has not yet been described outside of the Pacific, the completion of GTMP may identify other districts in which a similar mismatch between the prevalence of TF and TT could indicate that MDA might not be warranted. The approach taken here may encourage other nations to include operational surveillance of ocular *Ct* infection in trachoma elimination programmes. A combined approach such as ours would be optimal, but may be unrealistic on the global scale of trachoma elimination. Infection testing has been shown to be cost–effective in low-prevalence areas (135) and the data here demonstrate the high information gain that can be achieved through application of this highly specific marker of current ocular *Ct* infection. The introduction of at least one test for infection into the WHO guidelines for trachoma assessment, alongside clinical grading, may prevent rounds of MDA that could be otherwise directed at hyper-endemic districts.

9. REFERENCES

1. Köberlein J, Beifus K, Schaffert C, Finger RP. The economic burden of visual impairment and blindness: a systematic review. BMJ Open. 2013 Jan 1;3(11):e003471.
2. Bourne RA, Stevens GA, White RA, Smith JL, Flaxman SR, Price H, et al. Causes of vision loss worldwide, 1990-2010: a systematic analysis. Lancet Glob Heal. 2013;1(6):e339-49.
3. Frick KD, Basilion E V, Hanson CL, Colchero MA. Estimating the burden and economic impact of trachomatous visual loss. Ophthalmic Epidemiol. 2003 Apr;10(2):121–32.
4. World Health Organization. Report of the 1st meeting of the WHO Alliance for the elimination of trachoma. 30 June - 1 July. Geneva, Switzerland; 1997.
5. Taylor HR. Trachoma: a blinding scourge from the Bronze Age to the twenty-first century. 1st ed. East Melbourne: Centre for Eye Research, Australia; 2008.
6. Mathew AA, Keeffe JE, Le Mesurier RT, Taylor HR. Trachoma in the Pacific Islands: evidence from Trachoma Rapid Assessment. Br J Ophthalmol. 2009;93(7):866–70.
7. Grassly NC, Ward ME, Ferris S, Mabey DC, Bailey RL. The natural history of trachoma infection and disease in a Gambian cohort with frequent follow-up. PLoS Negl Trop Dis. Public Library of Science; 2008 Jan 2;2(12):e341.
8. Francis V, Turner V. Acheiving community support for trachoma control: a guide for district health work. Geneva, Switzerland: World Health Organization; 1995.
9. Wolle MA, Muñoz B, Mkocha H, West SK. Age, sex, and cohort effects in a longitudinal study of trachomatous scarring. Invest Ophthalmol Vis Sci. 2009;50(2):592–6.
10. Wolle MA, Muñoz BE, Mkocha H, West SK. Constant ocular infection with Chlamydia trachomatis predicts risk of scarring in children in Tanzania. Ophthalmology. 2009;116(2):243–7.
11. Hu VH, Massae P, Weiss HA, Chevallier C, Onyango JJ, Afwamba IA, et al. Bacterial infection in scarring trachoma. Invest Ophthalmol Vis Sci. 2011 Apr;52(5):2181–6.
12. Melese M, West ES, Alemayehu W, Munoz B, Worku A, Gaydos CA, et al. Characteristics of trichiasis patients presenting for surgery in rural Ethiopia. Br J Ophthalmol. 2005 Sep 1;89(9):1084–8.
13. Schlosser K. Trachoma through history. International Trachoma Initiative. New York, USA; 2004.
14. World Health Organization. Trachoma: fact sheet updated July 2016. http://www.who.int/mediacentre/factsheets/fs382/en/; accessed 30 July 2016. 2016.
15. Solomon AW, Pavluck AL, Courtright P, Aboe A, Adamu L, Alemayehu W, et al. The Global Trachoma Mapping Project: Methodology of a 34-Country Population-Based Study. Ophthalmic Epidemiol. 2015;22(3):214–25.
16. Emerson PM, Lindsay SW, Alexander N, Bah M, Dibba SM, Faal HB, et al. Role of flies and provision of latrines in trachoma control: cluster-randomised controlled trial. Lancet. 2004;363(9415):1093–8.
17. Emerson PM, Bailey RL, Mahdi OS, Walraven GE, Lindsay SW. Transmission ecology of the fly Musca sorbens, a putative vector of trachoma. Trans R Soc Trop Med Hyg.

2000;94(1):28–32.

18. Kim J, Lietman T. Elements of a dirty face as individual risk factors for trachoma, from a cluster-randomized trial in Niger. Ann Glob Heal. 2015;81 (1):10–1.
19. Hägi M, Schémann J-F, Mauny F, Momo G, Sacko D, Traoré L, et al. Active trachoma among children in Mali: Clustering and environmental risk factors. PLoS Negl Trop Dis. 2010 Jan;4(1):e583.
20. Katz J, Zeger SL, Tielsch JM. Village and Household Clustering of Xerophthalmia and Trachoma. Int J Epidemiol. 1988 Dec 1;17(4):865–9.
21. West SK, Muñoz B, Lynch M, Kayongoya A, Mmbaga BB, Taylor HR. Risk factors for constant, severe trachoma among preschool children in Kongwa, Tanzania. Am J Epidemiol. 1996 Jan 1;143(1):73–8.
22. Burton MJ, Holland MJ, Faal N, Aryee EAN, Alexander NDE, Bah M, et al. Which members of a community need antibiotics to control trachoma? Conjunctival Chlamydia trachomatis infection load in Gambian villages. Invest Ophthalmol Vis Sci. 2003 Oct;44(10):4215–22.
23. Broman AT, Shum K, Munoz B, Duncan DD, West SK. Spatial clustering of ocular chlamydial infection over time following treatment, among households in a village in Tanzania. Invest Ophthalmol Vis Sci. 2006 Jan;47(1):99–104.
24. Mpyet C, Lass BD, Yahaya HB, Solomon AW. Prevalence of and risk factors for trachoma in Kano state, Nigeria. PLoS One. 2012;7(7):e40421.
25. Emerson P, Bailey R, Walraven G, Lindsay S. Human and other faeces as breeding media of the trachoma vector Musca sorbens. Med Vet Entomol. 2001;15(3):314–20.
26. Last AR, Burr SE, Weiss HA, Harding-Esch EM, Cassama E, Nabicassa M, et al. Risk Factors for Active Trachoma and Ocular Chlamydia trachomatis Infection in Treatment-Naive Trachoma-Hyperendemic Communities of the Bijagos Archipelago, Guinea Bissau. PLoS Negl Trop Dis. 2014;8(6).
27. King JD, Odermatt P, Utzinger JJ, Ngondi J, Bamani S, Kamissoko Y, et al. Trachoma among children in community surveys from four African countries and implications of using school surveys for evaluating prevalence. Int Health. 2013 Dec;5(4):280–7.
28. Cruz AA V, Medina NH, Ibrahim MM, Souza RM, Gomes UA, Goncalves GFOR. Prevalence of trachoma in a population of the upper Rio Negro basin and risk factors for active disease. Ophthalmic Epidemiol. 2008;15(4):272–8.
29. Zambrano AI, Muñoz BE, Mkocha H, West SK. Exposure to an Indoor Cooking Fire and Risk of Trachoma in Children of Kongwa, Tanzania. PLoS Negl Trop Dis. 2015;9(6):e0003774.
30. Solomon AW, Holland MJ, Burton MJ, West SK, Alexander NDE, Aguirre A, et al. Strategies for control of trachoma: observational study with quantitative PCR. Lancet. 2003;362(9379):198–204.
31. Goldschmidt P, Benallaoua D, Amza A, Einterz E, Huguet P, Poisson F, et al. Clinical and Microbiological Assessment of Trachoma in the Kolofata Health District, Far North Region, Cameroon. Trop Med Health. 2012;40(1):7–14.

32. Schémann JF, Sacko D, Banou A, Bamani S, Boré B, Coulibaly S, et al. [Cartography of trachoma in Mali: results of a national survey]. Bull World Health Organ. 1998 Jan;76(6):599–606.
33. Bailey R, Duong T, Carpenter R, Whittle H, Mabey D. The duration of human ocular Chlamydia trachomatis infection is age dependent. Epidemiol Infect. 1999 Dec;123(3):479–86.
34. King J, Schindler C, Ngondi J, Odermatt P, Utzinger J, Mulualem A, et al. Impact of the SAFE strategy on trachomatous scarring among children in Ethiopia. In: Tropical Medicine and International Health, editor. Abstracts of the 9th European Congress on Tropical Medicine and International Health 6-10 September. Basel, Switzerland; 2015. p. 240.
35. West SK, Muñoz B, Mkocha H, Hsieh YH, Lynch MC. Progression of active trachoma to scarring in a cohort of Tanzanian children. Ophthalmic Epidemiol. 2001 Jul;8(2–3):137–44.
36. Ramadhani AM, Derrick T, Holland MJ, Burton MJ. Blinding Trachoma: Systematic Review of Rates and Risk Factors for Progressive Disease. PLoS Negl Trop Dis. 2016;10(8):e0004859.
37. Roper KG, Taylor HR. Comparison of clinical and photographic assessment of trachoma. Br J Ophthalmol. 2009 Jun;93(6):811–4.
38. Derrick T, Roberts C, Last AR, Burr SE, Holland MJ. Trachoma and Ocular Chlamydial Infection in the Era of Genomics. Mediators Inflamm. 2015;791847.
39. Burton MJ, Rajak SN, Hu VH, Ramadhani A, Habtamu E, Massae P, et al. Pathogenesis of progressive scarring trachoma in ethiopia and Tanzania and its implications for disease control: two cohort studies. PLoS Negl Trop Dis. 2015;9(5):e0003763.
40. Cevallos V, Whitcher JP, Melese M, Alemayehu W, Yi E, Chidambaram JD, et al. Association of conjunctival bacterial infection and female sex in cicatricial trachoma. Invest Ophthalmol Vis Sci. 2012 Aug;53(9):5208–12.
41. Cromwell EA, Courtright P, King JD, Rotondo LA, Ngondi J, Emerson PM. The excess burden of trachomatous trichiasis in women: a systematic review and meta-analysis. Trans R Soc Trop Med Hyg. 2009 Oct 1;103(10):985–92.
42. Habtamu E, Wondie T, Aweke S, Tadesse Z, Zerihun M, Zewdie Z, et al. Trachoma and Relative Poverty: A Case-Control Study. Small PLC, editor. PLoS Negl Trop Dis. 2015 Nov 23;9(11):e0004228.
43. Habtamu E, Wondie T, Aweke S, Tadesse Z, Zerihun M, Zewudie Z, et al. The Impact of Trachomatous Trichiasis on Quality of Life: A Case Control Study. PLoS Negl Trop Dis. 2015 Nov;9(11):e0004254.
44. King JD, Jip N, Jugu YS, Othman A, Rodgers AF, Dajom DY, et al. Mapping trachoma in Nasarawa and Plateau States, central Nigeria. Br J Ophthalmol. 2010 Jan;94(1):14–9.
45. Cromwell EA, Amza A, Kadri B, Beidou N, King JD, Sankara D, et al. Trachoma prevalence in Niger: results of 31 district-level surveys. Trans R Soc Trop Med Hyg. 2013 Nov 25;108(1):42–8.

46. Roba AA, Wondimu A, Patel D, Zondervan M. Effects of intervention with the SAFE strategy on trachoma across Ethiopia. J Epidemiol Community Health. BMJ Publishing Group Ltd; 2011 Aug 6;65(7):626–31.
47. Muhammad N, Mohammed A, Isiyaku S, Adamu MD, Gwom A, Rabiu MM. Mapping trachoma in 25 local government areas of Sokoto and Kebbi states, northwestern Nigeria. Br J Ophthalmol. 2014 Apr;98(4):432–7.
48. King JD, Richards F, Eigege A, Jip N, Umaru J, Deming M, et al. INTEGRATING PROTOCOLS FOR MAPPING TRACHOMA AND URINARY SCHISTOSOMIASIS. CAN SURVEYS BE DONE SIMULTANEOUSLY? Am J Trop Med Hyg. 2008;79(6):98–9.
49. Kalua K, Phiri M, Kumwenda I, Masika M, Pavluck AL, Willis R, et al. Baseline Trachoma Mapping in Malawi with the Global Trachoma Mapping Project (GTMP). Ophthalmic Epidemiol. 2015 Jun;22(3):176–83.
50. Karimurio J, Gichangi M, Ilako DR, Adala HS, Kilima P. Prevalence of trachoma in six districts of Kenya. East Afr Med J. 2006 Apr;83(4):63–8.
51. Jip NF, King JD, Diallo MO, Miri ES, Hamza AT, Ngondi J, et al. Blinding Trachoma in Katsina State, Nigeria: Population-Based Prevalence Survey in Ten Local Government Areas. Ophthalmic Epidemiol. 2008;15(5):294–302.
52. Noa Noatina B, Kagmeni G, Mengouo MN, Moungui HC, Tarini A, Zhang Y, et al. Prevalence of trachoma in the Far North region of Cameroon: results of a survey in 27 Health Districts. PLoS Negl Trop Dis. 2013 May;7(5):e2240.
53. Hassan A, Ngondi JM, King JD, Elshafie BE, Al Ginaid G, Elsanousi M, et al. The prevalence of blinding trachoma in northern states of Sudan. PLoS Negl Trop Dis. 2011 Jan;5(5):e1027.
54. Noatina BN, Kagmeni G, Mengouo MN, Moungui HC, Tarini A, Zhang Y, et al. Prevalence of Trachoma in the Far North Region of Cameroon: Results of a Survey in 27 Health Districts. PLoS Negl Trop Dis. 2013;7(5).
55. Ngondi J, Onsarigo A, Adamu L, Matende I, Baba S, Reacher M, et al. The epidemiology of trachoma in Eastern Equatoria and Upper Nile States, southern Sudan. Bull World Health Organ. 2005 Dec;83(12):904–12.
56. Koroma JB, Heck E, Vandy M, Sonnie M, Hodges M, MacArthur C, et al. The Epidemiology of Trachoma in the Five Northern Districts of Sierra Leone. Ophthalmic Epidemiol. 2011 Aug;18(4):150–7.
57. Quicke E, Sillah A, Harding-Esch EM, Last A, Joof H, Makalo P, et al. Follicular trachoma and trichiasis prevalence in an urban community in The Gambia, West Africa: is there a need to include urban areas in national trachoma surveillance? Trop Med Int Health. 2013 Sep 6;18(11):1344–52.
58. Astle WF, Wiafe B, Ingram AD, Mwanga M, Glassco CB. Trachoma Control in Southern Zambia—An International Team Project Employing the SAFE Strategy. Ophthalmic Epidemiol. 2006;13(4):227–36.
59. Ramyil A, Wade P, Ogoshi C, Goyol M, Adenuga O, Dami N, et al. Prevalence of Trachoma in Jigawa State, Northwestern Nigeria. Ophthalmic Epidemiol. 2015

Jun;22(3):184–9.

60. Mpyet C, Ogoshi C, Goyol M. Prevalence of trachoma in Yobe State, north-eastern Nigeria. Ophthalmic Epidemiol. 2008 Jan;15(5):303–7.
61. Kur LW, Picon D, Adibo O, Robinson E, Sabasio A, Edwards T, et al. Trachoma in Western Equatoria State, Southern Sudan: Implications for National Control. PLoS Negl Trop Dis. 2009;3(7).
62. Yalew KN, Mekonnen MG, Jemaneh AA. Trachoma and its determinants in Mojo and Lume districts of Ethiopia. Pan Afr Med J. 2012 Jan;13 Suppl 1:8.
63. Mansur R, Muhammad N, Liman IRN. Prevalence and magnitude of trachoma in a local government area of Sokoto State, north western Nigeria. Niger J Med. 2007 Jan;16(4):348–53.
64. Edwards KJ, Logan JMJ, Langham S, Swift C, Gharbia SE. Utility of real-time amplification of selected 16S rRNA gene sequences as a tool for detection and identification of microbial signatures directly from clinical samples. J Med Microbiol. 2012 May;61(Pt 5):645–52.
65. Dolin PJ, Faal H, Johnson GJ, Ajewole J, Mohamed AA, Lee PS. Trachoma in the Gambia. Br J Ophthalmol. 1998 Aug 1;82(8):930–3.
66. MacCallan AF. The epidemiology of trachoma. Br J Ophthalmol. 1931;15(7):369–411.
67. Thylefors B, Dawson CR, Jones BR, West SK, Taylor HR. A simple system for the assessment of trachoma and its complications. Bull World Health Organ. 1987;65(4):477–83.
68. Taylor HR, West SK, Katala S, Foster A. Trachoma - Evaluation of a new grading scheme in the United Republic of Tanzania. Bull World Health Organ. 1987;65(4):485–8.
69. Rahman SA, Yu SN, Amza A, Gebreselassie S, Kadri B, Baido N, et al. Reliability of Trachoma Clinical Grading-Assessing Grading of Marginal Cases. PLoS Negl Trop Dis. 2014;8(5).
70. Dawson CR, Jones BR, Tarizzo ML, World Health Organization. Guide to trachoma control in programmes for the prevention of blindness. Geneva, Switzerland: World Health Organization; 1981.
71. Michel C-E, Roper KG, Divena MA, Lee HH, Taylor HR. Correlation of clinical trachoma and infection in Aboriginal communities. PLoS Neglected Trop Dis. 2011 Jan;5(3):e986.
72. Sintayehu G, Zerihun T, Ayalew S, Yu SN, Stoller NE, Zhou ZX, et al. Inter-rater agreement between trachoma graders: comparison of grades given in field conditions versus grades from photographic review. Ophthalmic Epidemiol. 2015;22(3):162–9.
73. Solomon AW, Bowman RJC, Yorston D, Massae PA, Safari S, Savage B, et al. Operational evaluation of the use of photographs for grading active trachoma. Am J Trop Med Hyg. 2006;74(3):505–8.
74. Derrick T, Holland MJ, Cassama E, Markham-David R, Nabicassa M, Marks M, et al. Can corneal pannus with trachomatous inflammation - follicular be used in combination as an improved specific clinical sign for current ocular Chlamydia trachomatis infection? Parasit Vectors. 2016;9.

75. Ngondi J, Reacher M, Matthews F, Brayne C, Emerson P. Trachoma survey methods: a literature review. Bull World Health Organ. 2009 Feb;87(2):143–51.
76. Negrel A-D, Taylor HR, West SK. Guidelines for the rapid assessment of trachoma. Geneva, Switzerland; 2001.
77. Dolin PJ, Faal H, Johnson GJ, Minassian D, Sowa S, Day S, et al. Reduction of trachoma in a sub-Saharan village in absence of a disease control programme. Lancet. 1997 May 24;349(9064):1511–2.
78. Hoechsmann A, Metcalfe N, Kanjaloti S, Godia H, Mtambo O, Chipeta T, et al. Reduction of trachoma in the absence of antibiotic treatment: evidence from a population-based survey in Malawi. Ophthalmic Epidemiol. 2001 Jul;8(2–3):145–53.
79. Burton MJ, Holland MJ, Makalo P, Aryee EAN, Sillah A, Cohuet S, et al. Profound and sustained reduction in Chlamydia trachomatis in The Gambia: a five-year longitudinal study of trachoma endemic communities. PLoS Negl Trop Dis. 2010;4(10):10.
80. Chami Y, Hammou J, Mahjour J. Lessons from the moroccan national trachoma control programme. Community Eye Health. 2004 Dec;17(52):59.
81. Solomon AW, Harding-Esch E, Alexander NDE, Aguirre A, Holland MJ, Bailey RL, et al. Two doses of azithromycin to eliminate trachoma in a Tanzanian community. N Engl J Med. 2008 Apr 24;358(17):1870–1.
82. Yayemain D, King JD, Debrah O, Emerson PM, Aboe A, Ahorsu F, et al. Achieving trachoma control in Ghana after implementing the SAFE strategy. Trans R Soc Trop Med Hyg. 2009 Oct 1;103(10):993–1000.
83. World Health Organization. Report of the 2nd Global Scientific Meeting on Trachoma. 25-27 August. Geneva, Switzerland; 2003.
84. Habtamu E, Wondie T, Aweke S, Tadesse Z, Zerihun M, Zewudie Z, et al. Posterior lamellar versus bilamellar tarsal rotation surgery for trachomatous trichiasis in Ethiopia: a randomised controlled trial. Lancet Glob Heal. 2016;4(3):e175-84.
85. West ES, Mkocha H, Munoz B, Mabey D, Foster A, Bailey R, et al. Risk factors for postsurgical trichiasis recurrence in a trachoma-endemic area. Invest Ophthalmol Vis Sci. 2005 Feb;46(2):447–53.
86. Burton MJ, Rajak SN, Ramadhani A, Weiss HA, Habtamu E, Abera B, et al. Post-operative recurrent trachomatous trichiasis is associated with increased conjunctival expression of S100A7 (psoriasin). PLoS Negl Trop Dis. 2012 Jan;6(12):e1985.
87. West S, Alemayehu W, Munoz B, Gower E. Azithromycin prevents recurrence of severe trichiasis following trichiasis surgery: STAR trial. Ophthalmic Epidemiol. 2007;14(5):273–7.
88. Burton MJ, Kinteh F, Jallow O, Sillah A, Bah M, Faye M, et al. A randomised controlled trial of azithromycin following surgery for trachomatous trichiasis in the Gambia. Br J Ophthalmol. 2005 Oct;89(10):1282–8.
89. Habtamu E, Rajak SN, Tadesse Z, Wondie T, Zerihun M, Guadie B, et al. Epilation for Minor Trachomatous Trichiasis: Four-Year Results of a Randomised Controlled Trial. PLoS Negl Trop Dis. 2015;9(3):1–15.

90. Rajak SN, Habtamu E, Weiss HA, Bedri A, Zerihun M, Gebre T, et al. Why Do People Not Attend for Treatment for Trachomatous Trichiasis in Ethiopia? A Study of Barriers to Surgery. PLoS Negl Trop Dis. 2012;6(8).
91. World Health Organization. Report of the 3rd Global Scientific Meeting on Trachoma. 19-20 July. Johns Hopkins University, Baltimore, MA; 2010.
92. Pasquale T, Tan J. Nonantimicrobial effects of antibacterial agents. Clin Infect Dis. 2005;40(1):127–35.
93. Whitty CJM, Glasgow KW, Sadiq ST, Mabey DC, Bailey R. Impact of community-based mass treatment for trachoma with oral azithromycin on general morbidity in Gambian children. Pediatr Infect Dis J. 1999;18(11):955–8.
94. Fraser-Hurt N, Bailey RL, Cousens S, Mabey DCW, Faal H, Mabey DCW. Efficacy of oral azithromycin versus topical tetracycline in mass treatment of endemic trachoma. Bull World Health Organ. 2001;79(7):632–40.
95. Bowman RJ, Sillah A, Van Dehn C, Goode VM, Muqit MM, Muquit M, et al. Operational comparison of single-dose azithromycin and topical tetracycline for trachoma. Invest Ophthalmol Vis Sci. 2000 Dec;41(13):4074–9.
96. Schachter J, West SK, Mabey D, Dawson CR, Bobo L, Bailey R, et al. Azithromycin in control of trachoma. Lancet. 1999 Aug 21;354(9179):630–5.
97. Gebre T, Ayele B, Zerihun M, Genet A, Stoller NE, Zhou Z, et al. Comparison of annual versus twice-yearly mass azithromycin treatment for hyperendemic trachoma in Ethiopia: a cluster-randomised trial. Lancet. 2012;379(9811):143–51.
98. West SK, Bailey R, Munoz B, Edwards T, Mkocha H, Gaydos C, et al. A Randomized Trial of Two Coverage Targets for Mass Treatment with Azithromycin for Trachoma. PLoS Negl Trop Dis. 2013;7(8).
99. Solomon AW, Holland MJ, Alexander NDE, Massae PA, Aguirre A, Natividad-Sancho A, et al. Mass treatment with single-dose azithromycin for trachoma. N Engl J Med. 2004 Nov 4;351(19):1962–71.
100. King JD. Novel approaches to evaluate the impact of the SAFE strategy on trachoma and other neglected tropical diseases in Amhara National Regional State, Ethiopia [Doctoral thesis]. Faculty of Science, University of Basel; 2014.
101. Marks M, Bottomley C, Tome H, Pitakaka R, Butcher R, Sokana O, et al. Mass drug administration of azithromycin for trachoma reduces the prevalence of genital Chlamydia trachomatis infection in the Solomon Islands. Sex Transm Infect. 2016 Feb 17;
102. Marks M, Vahi V, Sokana O, Chi K-HH, Puiahi E, Kilua G, et al. Impact of community mass treatment with azithromycin for trachoma elimination on the prevalence of yaws. PLoS Negl Trop Dis. 2015;9(8):e0003988.
103. Hart JD, Edwards T, Burr SE, Harding-Esch EM, Takaoka K, Holland MJ, et al. Effect of azithromycin mass drug administration for trachoma on spleen rates in Gambian children. Trop Med Int Heal. 2014;19(2):207–11.
104. Coles CL, Mabula K, Seidman JC, Levens J, Mkocha H, Munoz B, et al. Mass Distribution of Azithromycin for Trachoma Control Is Associated With Increased Risk of

Azithromycin-Resistant Streptococcus pneumoniae Carriage in Young Children 6 Months After Treatment. Clin Infect Dis. 2013 Mar 13;56(11):1519–26.

105. Coles CL, Seidman JC, Levens J, Mkocha H, Munoz B, West S. Association of Mass Treatment with Azithromycin in Trachoma-Endemic Communities with Short-Term Reduced Risk of Diarrhea in Young Children. Am J Trop Med Hyg. 2011;85(4):691–6.
106. Porco TC, Gebre T, Ayele B, House J, Keenan J, Zhou Z, et al. Effect of mass distribution of azithromycin for trachoma control on overall mortality in Ethiopian children: a randomized trial. JAMA. 2009;302(9):962–8.
107. West SK, Moncada J, Munoz B, Mkocha H, Storey P, Hardick J, et al. Is There Evidence for Resistance of Ocular Chlamydia trachomatis to Azithromycin after Mass Treatment for Trachoma Control? J Infect Dis. 2014 Jan 19;210(1):65–71.
108. Seidman JC, Coles CL, Silbergeld EK, Levens J, Mkocha H, Johnson LB, et al. Increased carriage of macrolide-resistant fecal E. coli following mass distribution of azithromycin for trachoma control. Int J Epidemiol. 2014 Aug;43(4):1105–13.
109. Skalet AH, Cevallos V, Ayele B, Gebre T, Zhou Z, Jorgensen JH, et al. Antibiotic Selection Pressure and Macrolide Resistance in Nasopharyngeal Streptococcus pneumoniae: A Cluster-Randomized Clinical Trial. PLoS Med. 2010;7(12).
110. Coles CL, Levens J, Seidman JC, Mkocha H, Munoz B, West S. Mass Distribution of Azithromycin for Trachoma Control Is Associated With Short-term Reduction in Risk of Acute Lower Respiratory Infection in Young Children. Pediatr Infect Dis J. 2012;31(4):341–6.
111. Batt SL, Charalambous BM, Solomon AW, Knirsch C, Massae PA, Safari S, et al. Impact of azithromycin administration for trachoma control on the carriage of antibiotic-resistant Streptococcus pneumoniae. Antimicrob Agents Chemother. 2003 Sep;47(9):2765–9.
112. Keenan JD, Klugman KP, McGee L, Vidal JE, Chochua S, Hawkins P, et al. Evidence for clonal expansion after antibiotic selection pressure: Pneumococcal multilocus sequence types before and after mass azithromycin treatments. J Infect Dis. 2015;211(6):988–94.
113. Haug S, Lakew T, Habtemariam G, Alemayehu W, Cevallos V, Zhou Z, et al. The decline of pneumococcal resistance after cessation of mass antibiotic distributions for trachoma. Clin Infect Dis. 2010 Sep 1;51(5):571–4.
114. Brunham RC, Rekart ML. The Arrested Immunity Hypothesis and the Epidemiology of Chlamydia Control. Sex Transm Dis. 2008 Jan;35(1):53–4.
115. Burton MJ, Holland MJ, Makalo P, Aryee EAN, Alexander NDE, Sillah A, et al. Re-emergence of Chlamydia trachomatis infection after mass antibiotic treatment of a trachoma-endemic Gambian community: a longitudinal study. Lancet. 2005 Jan;365(9467):1321–8.
116. West SK, Munoz BE, Mkocha H, Gaydos C, Quinn T. Risk of Infection with Chlamydia trachomatis from Migrants to Communities Undergoing Mass Drug Administration for Trachoma Control. Ophthalmic Epidemiol. 2015 Jun;22(3):170–5.
117. Ejere HOD, Alhassan MB, Rabiu M, Ejere Henry OD, Alhassan Mahmoud B, Rabiu M, et al. Face washing promotion for preventing active trachoma. Cochrane Database Syst

Rev. John Wiley & Sons, Ltd; 2015;(2).

118. West S, Muñoz B, Lynch M, Kayongoya A, Chilangwa Z, Mmbaga BBO, et al. Impact of face-washing on trachoma in Kongwa, Tanzania. Lancet. 1995;345(8943):155–8.
119. Stocks ME, Ogden S, Haddad D, Addiss DG, McGuire C, Freeman MC. Effect of Water, Sanitation, and Hygiene on the Prevention of Trachoma: A Systematic Review and Meta-Analysis. PLoS Med. 2014;11(2):e1001605.
120. Courtright P, Sheppard J, Lane S, Sadek A, Schachter J, Dawson CR. Latrine ownership as a protective factor in inflammatory trachoma in Egypt. Br J Ophthalmol. 1991;75(6):322–5.
121. Stoller NE, Gebre T, Ayele B, Zerihun M, Assefa Y, Habte D, et al. Efficacy of latrine promotion on emergence of infection with ocular Chlamydia trachomatis after mass antibiotic treatment: A cluster-randomized trial. Vol. 3, International Health. 2011. p. 75–84.
122. Collier L, Duke-Elder S, Jones B. Experimental trachoma produced by cultured virus. Br J Ophthalmol. 1958;42(12):705–20.
123. Tang FF, Huang YT, Chang HL, Wong KC. Isolation of trachoma virus in chick embryo. J Hyg Epidemiol Microbiol Immunol. 1957;1(2):109–20.
124. Macchiavello A. The Virus of Trachoma and its Cultivation in the Yolk Sac of the Hen's Egg. Rev Ecuat Hig Med Trop. 1944;1(2):33.
125. Bastidas RJ, Elwell CA, Engel JN, Valdivia RH. Chlamydial intracellular survival strategies. Cold Spring Harb Perspect Med. 2013 May 1;3(5):a010256.
126. Ramadhani AM, Derrick T, Macleod D, Holland MJ, Burton MJ. The Relationship between Active Trachoma and Ocular Chlamydia trachomatis Infection before and after Mass Antibiotic Treatment. PLoS Negl Trop Dis. 2016;10(10).
127. Thein J, Zhao PN, Liu HS, Xu JJ, Jha HC, Miao YH, et al. Does clinical diagnosis indicate ocular chlamydial infection in areas with a low prevalence of trachoma? Ophthalmic Epidemiol. 2002;9(4):263–9.
128. Baral K, Osaki S, Shreshta B, Panta CR, Boulter A, Pang F, et al. Reliability of clinical diagnosis in identifying infectious trachoma in a low-prevalence area of Nepal. Bull World Health Organ. 1999 Jan;77(6):461–6.
129. Burton MJ, Hu VH, Massae P, Burr SE, Chevallier C, Afwamba IA, et al. What is causing active trachoma? The role of nonchlamydial bacterial pathogens in a low prevalence setting. Invest Ophthalmol Vis Sci. 2011 Jul;52(8):6012–7.
130. Lee JS, Muñoz BE, Mkocha H, Gaydos CA, Quinn TC, West SK, et al. The Effect of Multiple Rounds of Mass Drug Administration on the Association between Ocular Chlamydia trachomatis Infection and Follicular Trachoma in Preschool-Aged Children. PLoS Negl Trop Dis. 2014;8(4):e2761.
131. Gower EW, Solomon AW, Burton MJ, Aguirre A, Muñoz B, Bailey R, et al. Chlamydial positivity of nasal discharge at baseline is associated with ocular chlamydial positivity 2 months following azithromycin treatment. Invest Ophthalmol Vis Sci. 2006 Nov;47(11):4767–71.

132. West S, Munoz B, Bobo L, Quinn TC, Mkocha H, Lynch M, et al. Nonocular Chlamydia infection and risk of ocular reinfection after mass treatment in a trachoma-endemic area. Invest Ophthalmol Vis Sci. 1993;34(11):3194–8.
133. Ramesh A, Kovats S, Haslam D, Schmidt E, Gilbert CE. The Impact of Climatic Risk Factors on the Prevalence, Distribution, and Severity of Acute and Chronic Trachoma. PLoS Negl Trop Dis. 2013 Nov;7(11):e2513.
134. Harding-Esch EM, Sillah A, Edwards T, Burr SE, Hart JD, Joof H, et al. Mass treatment with azithromycin for trachoma: when is one round enough? Results from the PRET Trial in the Gambia.[Erratum appears in PLoS Negl Trop Dis. 2013 Jun;7(6). doi:10.1371/annotation/0bae8b34-5ae7-4044-a071-8d88d520a01b]. PLoS Negl Trop Dis. 2013;7(6):e2115.
135. Harding-Esch E, Jofre-Bonet M, Dhanjal JK, Burr S, Edwards T, Holland M, et al. Costs of Testing for Ocular Chlamydia trachomatis Infection Compared to Mass Drug Administration for Trachoma in The Gambia: Application of Results from the PRET Study. PLoS Negl Trop Dis. 2015 Apr;9(4):e0003670.
136. Last AR, Burr SE, Weiss HA, Harding-Esch EM, Cassama E, Nabicassa M, et al. Risk factors for active trachoma and ocular Chlamydia trachomatis infection in treatment-naïve trachoma-hyperendemic communities of the Bijagós Archipelago, Guinea Bissau. PLoS Negl Trop Dis. 2014 Jun;8(6):e2900.
137. Yohannan J, Munoz B, Mkocha H, Gaydos CA, Bailey R, Lietman TA, et al. Can we stop mass drug administration prior to 3 annual rounds in communities with low prevalence of trachoma?: PRET Ziada trial results. JAMA Ophthalmol. 2013 Apr;131(4):431–6.
138. Harding-Esch EM, Edwards T, Sillah A, Sarr I, Roberts CH, Snell P, et al. Active Trachoma and Ocular Chlamydia trachomatis Infection in Two Gambian Regions: On Course for Elimination by 2020? PLoS Negl Trop Dis. 2009 Jan;3(12):e573.
139. Bailey RL, Hampton TJ, Hayes LJ, Ward ME, Whittle HC, Mabey DC. Polymerase chain reaction for the detection of ocular chlamydial infection in trachoma-endemic communities. J Infect Dis. 1994 Sep;170(3):709–12.
140. Amza A, Kadri B, Nassirou B, Stoller NE, Yu SN, Zhou Z, et al. A Cluster-Randomized Controlled Trial Evaluating the Effects of Mass Azithromycin Treatment on Growth and Nutrition in Niger. Am J Trop Med Hyg. 2013;88(1):138–43.
141. Amza A, Kadri B, Nassirou B, Stoller NE, Yu SN, Zhou Z, et al. Community risk factors for ocular Chlamydia infection in Niger: pre-treatment results from a cluster-randomized trachoma trial. PLoS Negl Trop Dis. 2012 Jan;6(4):e1586.
142. Yohannan J, He B, Wang J, Greene G, Schein Y, Mkocha H, et al. Geospatial distribution and clustering of Chlamydia trachomatis in communities undergoing mass azithromycin treatment. Invest Ophthalmol Vis Sci. 2014 Jul;55(7):4144–50.
143. West SK, Munoz B, Mkocha H, Holland MJ, Aguirre A, Solomon AW, et al. Infection with Chlamydia trachomatis after mass treatment of a trachoma hyperendemic community in Tanzania: a longitudinal study. Lancet. 2005 Oct 8;366(9493):1296–300.
144. Abdou A, Nassirou B, Kadri B, Moussa F, Munoz BE, Opong E, et al. Prevalence and

risk factors for trachoma and ocular Chlamydia trachomatis infection in Niger. Br J Ophthalmol. 2007;91(1):13–7.

145. Keenan JD, Moncada J, Gebre T, Ayele B, Chen MC, Yu SN, et al. Chlamydial infection during trachoma monitoring: are the most difficult-to-reach children more likely to be infected? Trop Med Int Health. 2012 Mar;17(3):392–6.
146. Bird M, Dawson CR, Schachter JS, Miao Y, Shama A, Osman A, et al. Does the diagnosis of trachoma adequately identify ocular chlamydial infection in trachoma-endemic areas? J Infect Dis. 2003 May 15;187(10):1669–73.
147. Yang JL, Hong KC, Schachter J, Moncada J, Lekew T, House JI, et al. Detection of Chlamydia trachomatis Ocular Infection in Trachoma-Endemic Communities by rRNA Amplification. Invest Ophthalmol Vis Sci. 2009;50(1):90–4.
148. House JI, Ayele B, Porco TC, Zhou Z, Hong KC, Gebre T, et al. Assessment of herd protection against trachoma due to repeated mass antibiotic distributions: a cluster-randomised trial. Lancet (London, England). 2009 Mar 28;373(9669):1111–8.
149. See CW, Alemayehu W, Melese M, Zhou Z, Porco TC, Shiboski S, et al. How Reliable Are Tests for Trachoma?—A Latent Class Approach. Investig Opthalmology Vis Sci. 2011;52(9):6133–7.
150. Melese M, Yi E, Cevallos V, Ray K, Hong KC, Porco TC, et al. Comparison of Annual and Biannual Mass Antibiotic Administration for Elimination of Infectious Trachoma. J Am Med Assoc. 2008;299(7):778–84.
151. Miller K, Schmidt G, Melese M, Alemayehu W, Yi E, Cevallos V, et al. How reliable is the clinical exam in detecting ocular chlamydial infection? Ophthalmic Epidemiol. 2004;11(3):255–62.
152. Stephens RS, Tam MR, Kuo CC, Nowinski RC. Monoclonal antibodies to Chlamydia trachomatis: antibody specificities and antigen characterization. J Immunol. 1982 Mar;128(3):1083–9.
153. Wang SP, Kuo CC, Barnes RC, Stephens RS, Grayston JT. Immunotyping of Chlamydia trachomatis with monoclonal antibodies. J Infect Dis. 1985 Oct;152(4):791–800.
154. Brunelle BW, Sensabaugh GF. The ompA gene in Chlamydia trachomatis differs in phylogeny and rate of evolution from other regions of the genome. Infect Immun. 2006 Jan;74(1):578–85.
155. Klint M, Fuxelius H-H, Goldkuhl RR, Skarin H, Rutemark C, Andersson SGE, et al. High-resolution genotyping of Chlamydia trachomatis strains by multilocus sequence analysis. J Clin Microbiol. 2007 May;45(5):1410–4.
156. Pannekoek Y, Morelli G, Kusecek B, Morré SA, Ossewaarde JM, Langerak AA, et al. Multi locus sequence typing of Chlamydiales: clonal groupings within the obligate intracellular bacteria Chlamydia trachomatis. BMC Microbiol. 2008 Jan;8:42.
157. Wang Y, Skilton RJ, Cutcliffe LT, Andrews E, Clarke IN, Marsh P. Evaluation of a high resolution genotyping method for Chlamydia trachomatis using routine clinical samples. PLoS One. 2011 Jan;6(2):e16971.
158. Fehlner-Gardiner C, Roshick C, Carlson JH, Hughes S, Belland RJ, Caldwell HD, et al.

Molecular basis defining human Chlamydia trachomatis tissue tropism. A possible role for tryptophan synthase. J Biol Chem. 2002 Jul 26;277(30):26893–903.

159. Akers JC, Tan M. Molecular mechanism of tryptophan-dependent transcriptional regulation in Chlamydia trachomatis. J Bacteriol. 2006 Jun;188(12):4236–43.
160. Andersson P, Harris SR, Smith HMBS, Hadfield J, O'Neill C, Cutcliffe LT, et al. Chlamydia trachomatis from Australian Aboriginal people with trachoma are polyphyletic composed of multiple distinctive lineages. Nat Commun. 2016;7:10688.
161. Stephens RS, Kalman S, Lammel C, Fan J, Marathe R, Aravind L, et al. Genome sequence of an obligate intracellular pathogen of humans: Chlamydia trachomatis. Science. 1998 Oct 23;282(5389):754–9.
162. Last AR, Roberts CH, Cassama E, Nabicassa M, Molina-Gonzalez S, Burr SE, et al. Plasmid copy number and disease severity in naturally occurring ocular Chlamydia trachomatis infection. J Clin Microbiol. 2013 Nov 6;52(1):324.
163. Nunes A, Gomes JP. Evolution, phylogeny, and molecular epidemiology of Chlamydia. Infect Genet Evol. 2014 Feb 5;
164. Gomes JP, Bruno WJ, Nunes A, Santos N, Florindo C, Borrego MJ, et al. Evolution of Chlamydia trachomatis diversity occurs by widespread interstrain recombination involving hotspots. Genome Res. 2007 Jan;17(1):50–60.
165. Jeffrey BM, Suchland RJ, Quinn KL, Davidson JR, Stamm WE, Rockey DD. Genome sequencing of recent clinical Chlamydia trachomatis strains identifies loci associated with tissue tropism and regions of apparent recombination. Infect Immun. 2010 Jun;78(6):2544–53.
166. Harris SR, Clarke IN, Seth-Smith HMB, Solomon AW, Cutcliffe LT, Marsh P, et al. Whole-genome analysis of diverse Chlamydia trachomatis strains identifies phylogenetic relationships masked by current clinical typing. Nat Genet. 2012 Apr;44(4):413–9, S1.
167. Christiansen MT, Brown AC, Kundu S, Tutill HJ, Williams R, Brown JR, et al. Whole-genome enrichment and sequencing of Chlamydia trachomatis directly from clinical samples. BMC Infect Dis. 2014 Jan;14:591.
168. Seth-Smith HMB, Harris SR, Skilton RJ, Radebe FM, Golparian D, Shipitsyna E, et al. Whole-genome sequences of Chlamydia trachomatis directly from clinical samples without culture. Genome Res. 2013 May;23(5):855–66.
169. Nunes A, Borrego MJ, Nunes B, Florindo C, Gomes JP. Evolutionary dynamics of ompA, the gene encoding the Chlamydia trachomatis key antigen. J Bacteriol. 2009 Dec;191(23):7182–92.
170. Gomes JP, Nunes A, Bruno WJ, Borrego MJ, Florindo C, Dean D. Polymorphisms in the nine polymorphic membrane proteins of Chlamydia trachomatis across all serovars: evidence for serovar Da recombination and correlation with tissue tropism. J Bacteriol. 2006 Jan;188(1):275–86.
171. Geisler WM, Suchland RJ, Rockey DD, Stamm WE. Epidemiology and clinical manifestations of unique Chlamydia trachomatis isolates that occupy nonfusogenic inclusions. J Infect Dis. 2001 Oct 1;184(7):879–84.

172. Carlson JH, Hughes S, Hogan D, Cieplak G, Sturdevant DE, McClarty G, et al. Polymorphisms in the Chlamydia trachomatis cytotoxin locus associated with ocular and genital isolates. Infect Immun. 2004 Dec;72(12):7063–72.
173. Sigar IM, Schripsema JH, Wang Y, Clarke IN, Cutcliffe LT, Seth-Smith HMB, et al. Plasmid deficiency in urogenital isolates of Chlamydia trachomatis reduces infectivity and virulence in a mouse model. Pathog Dis. 2014 Feb;70(1):61–9.
174. Ripa T, Nilsson PA. A Chlamydia trachomatis strain with a 377-bp deletion in the cryptic plasmid causing false-negative nucleic acid amplification tests. Sex Transm Dis. 2007 May;34(5):255–6.
175. Stothard DR, Williams JA, Van Der Pol B, Jones RB. Identification of a Chlamydia trachomatis serovar E urogenital isolate which lacks the cryptic plasmid. Infect Immun. 1998 Dec;66(12):6010–3.
176. Comanducci M, Ricci S, Cevenini R, Ratti G. Diversity of the Chlamydia trachomatis common plasmid in biovars with different pathogenicity. Plasmid. 1990 Mar;23(2):149–54.
177. Kari L, Whitmire WM, Olivares-Zavaleta N, Goheen MM, Taylor LD, Carlson JH, et al. A live-attenuated chlamydial vaccine protects against trachoma in nonhuman primates. J Exp Med. 2011 Oct 24;208(11):2217–23.
178. Kari L, Whitmire WM, Carlson JH, Crane DD, Reveneau N, Nelson DE, et al. Pathogenic diversity among Chlamydia trachomatis ocular strains in nonhuman primates is affected by subtle genomic variations. J Infect Dis. 2008 Feb 1;197(3):449–56.
179. Zhou Y, Gao H, Mihindukulasuriya KA, La Rosa PS, Wylie KM, Vishnivetskaya T, et al. Biogeography of the ecosystems of the healthy human body. Genome Biol. 2013 Jan 14;14(1):R1.
180. Dong Q, Brulc JM, Iovieno A, Bates B, Garoutte A, Miller D, et al. Diversity of bacteria at healthy human conjunctiva. Invest Ophthalmol Vis Sci. 2011 Jul;52(8):5408–13.
181. Huang Y, Yang B, Li W. Defining the normal core microbiome of conjunctival microbial communities. Clin Microbiol Infect. 2016;22(7):643.e7-643.e12.
182. Kugadas A, Gadjeva M. Impact of Microbiome on Ocular Health. Ocul Surf. 2016 Jul;14(3):342–9.
183. Doan T, Akileswaran L, Andersen D, Johnson B, Ko N, Shrestha A, et al. Paucibacterial Microbiome and Resident DNA Virome of the Healthy Conjunctiva. Investig Opthalmology Vis Sci. 2016;57(13):5116.
184. Shin H, Price K, Albert L, Dodick J, Park L, Dominguez-Belloa MG. Changes in the eye microbiota associated with contact lens wearing. MBio. 2016;7(2):1–6.
185. de Paiva CS, Jones DB, Stern ME, Bian F, Moore QL, Corbiere S, et al. Altered Mucosal Microbiome Diversity and Disease Severity in Sjögren Syndrome. Sci Rep. 2016;6:23561.
186. Zhou Y, Holland MJ, Makalo P, Joof H, Roberts CH, Mabey D, et al. The conjunctival microbiome in health and trachomatous disease: a case control study. Genome Med. 2014;6(11):99.

187. Lietman T, Brooks D, Moncada J, Schachter J, Dawson C, Dean D. Chronic follicular conjunctivitis associated with Chlamydia psittaci or Chlamydia pneumoniae. Clin Infect Dis. 1998 Jun;26(6):1335–40.
188. Dean D, Rothschild J, Ruettger A, Kandel RP, Sachse K. Zoonotic Chlamydiaceae species associated with trachoma, Nepal. Emerg Infect Dis. 2013 Dec;19(12):1948–55.
189. Burr SE, Hart JD, Edwards T, Baldeh I, Bojang E, Harding-Esch EM, et al. Association between ocular bacterial carriage and follicular trachoma following mass azithromycin distribution in The Gambia. PLoS Negl Trop Dis. 2013 Jul;7(7):e2347.
190. Taylor HR, Kolarczyk RA, Johnson SL, Schachter J, Prendergast RA. Effect of bacterial secondary infection in an animal model of trachoma. Infect Immun. 1984 Jun;44(3):614–6.
191. Høvding G. Acute bacterial conjunctivitis. Acta Ophthalmol. 2008;86(1):5–17.
192. Pettigrew MM, Laufer AS, Gent JF, Kong Y, Fennie KP, Metlay JP. Upper respiratory tract microbial communities, acute otitis media pathogens, and antibiotic use in healthy and sick children. Appl Environ Microbiol. 2012 Sep;78(17):6262–70.
193. Schornack MM, Siemsen DW, Bradley EA, Salomao DR, Lee HB. Ocular manifestations of molluscum contagiosum. Clin Exp Optom. 2006 Nov;89(6):390–3.
194. Magnus JA. Unilateral follicular conjunctivitis due to molluscum contagiosum. Br J Ophthalmol. 1944 May;28(5):245–8.
195. Thygeson P. Etiology and differential diagnosis of non-trachomatous follicular conjunctivitis. Bull World Health Organ. 1957 Jan;16(5):995–1011.
196. Darougar S, Quinlan MP, Gibson JA, Jones BR. Epidemic keratoconjunctivitis and chronic papillary conjunctivitis in London due to adenovirus type 19. Br J Ophthalmol. 1977 Feb;61(2):76–85.
197. Aoki K, Kaneko H, Kitaichi N, Ohguchi T, Tagawa Y, Ohno S. Clinical features of adenoviral conjunctivitis at the early stage of infection. Jpn J Ophthalmol. 2011 Jan;55(1):11–5.
198. Kuo IC, Espinosa C, Forman M, Pehar M, Maragakis LL, Valsamakis A. Detection and Prevalence of Adenoviral Conjunctivitis among Hospital Employees Using Real-Time Polymerase Chain Reaction as an Infection Prevention Tool. Infect Control Hosp Epidemiol. 2014 Jun;35(6):728–31.
199. Tullo AB. Clinical and epidemiological features of adenovirus keratoconjunctivitis. Trans Ophthalmol Soc U K. 1980 Jul;100(Pt 2):263–7.
200. Chang CH, Sheu MM, Lin KH, Chen CW. Hemorrhagic viral keratoconjunctivitis in Taiwan caused by adenovirus types 19 and 37: applicability of polymerase chain reaction-restriction fragment length polymorphism in detecting adenovirus genotypes. Cornea. 2001 Apr;20(3):295–300.
201. Boto-de-Los-Bueis A, Romero Gómez MP, Del Hierro Zarzuelo A, Sanchez EG, Mediero S, Noval S. Recurrent Ocular Surface Inflammation Associated With Human Herpesvirus 6 Infection. Eye Contact Lens. 2013 Nov 27;
202. Darougar S, Wishart MS, Viswalingam ND. Epidemiological and clinical features of

primary herpes simplex virus ocular infection. Br J Ophthalmol. 1985 Jan;69(1):2–6.

203. Uchio E, Takeuchi S, Itoh N, Matsuura N, Ohno S, Aoki K. Clinical and epidemiological features of acute follicular conjunctivitis with special reference to that caused by herpes simplex virus type 1. Br J Ophthalmol. 2000 Sep;84(9):968–72.
204. Hales RH, Ostler HB. Newcastle disease conjunctivitis with subepithelial infiltrates. Br J Ophthalmol. 1973 Sep;57(9):694–7.
205. Kayikçioglu O, Kir E, Söyler M, Güler C, Irkeç M. Ocular findings in a measles epidemic among young adults. Ocul Immunol Inflamm. 2000 Mar;8(1):59–62.
206. Hara J, Fujimoto F, Ishibashi T, Seguchi T, Nishimura K. Ocular manifestations of the 1976 rubella epidemic in Japan. Am J Ophthalmol. 1979 May;87(5):642–5.
207. Deckard PS, Bergstrom TJ. Rubeola keratitis. Ophthalmology. 1981 Aug;88(8):810–3.
208. Pavlopoulos GP, Giannakos GI, Theodosiadis PG, Moschos MM, Iliakis EK, Theodosiadis GP. Rubeola keratitis: a photographic study of corneal lesions. Cornea. 2008 May;27(4):411–6.
209. Riffenburgh R. Ocular manifestations of mumps. Arch Ophthalmol. 1961 Nov;66:739–43.
210. Meyer RF, Sullivan JH, Oh JO. Mumps conjunctivitis. Am J Ophthalmol. 1974 Dec;78(6):1022–4.
211. Triki H, Rezig D, Bahri O, Ben Ayed N, Ben Yahia A, Sadraoui A, et al. Molecular characterisation of a coxsackievirus A24 that caused an outbreak of acute haemorrhagic conjunctivitis, Tunisia 2003. Clin Microbiol Infect. 2007 Feb;13(2):176–82.
212. Wang M, Lu X, Hu A, Zhang M, Li X, Deng S, et al. Etiological characteristics of chlamydia trachoma conjunctivitis of Primary Boarding School students in the Qinghai Tibetan area. Sci China Life Sci. 2016 Jun 26;59(6):555–60.
213. Burton MJ, Bailey RL, Jeffries D, Rajak SN, Adegbola RA, Sillah A, et al. Conjunctival expression of matrix metalloproteinase and proinflammatory cytokine genes after trichiasis surgery. Invest Ophthalmol Vis Sci. 2010 Jul;51(7):3583–90.
214. Dean D, Kandel RP, Adhikari HK, Hessel T. Multiple Chlamydiaceae species in trachoma: implications for disease pathogenesis and control. PLoS Med. Public Library of Science; 2008 Jan 3;5(1):e14.
215. Burton MJ, Adegbola RA, Kinteh F, Ikumapayi UN, Foster A, Mabey DCW, et al. Bacterial infection and trachoma in the gambia: a case control study. Invest Ophthalmol Vis Sci. 2007 Oct;48(10):4440–4.
216. Chern KC, Shrestha SK, Cevallos V, Dhami HL, Tiwari P, Chern L, et al. Alterations in the conjunctival bacterial flora following a single dose of azithromycin in a trachoma endemic area. Br J Ophthalmol. 1999 Dec;83(12):1332–5.
217. Yoneda C, Dawson CR, Daghfous T, Hoshiwara I, Jones P, Messadi M, et al. Cytology as a guide to presence of chlamydial inclusions in Giemsa- stained conjunctival smears in severe endemic trachoma. Br J Ophthalmol. 1975;59(3):116–23.
218. Salim AR, Sheikh HA. Trachoma in the Sudan. A laboratory study. Br J Ophthalmol. BMJ Group; 1975 Aug;59(8):435–8.
219. Vastine DW, Dawson CR, Daghfous T, Messadi M, Hoshiwara I, Yoneda C, et al.

Severe endemic trachoma in Tunisia. I. Effect of topical chemotherapy on conjunctivitis and ocular bacteria. Br J Ophthalmol. 1974 Oct;58(10):833–42.

220. Reinhards J, Weber A, Nizetic B, Kupka K, Maxwell-Lyons F. Studies in the epidemiology and control of seasonal conjunctivitis and trachoma in southern Morocco. Bull World Health Organ. 1968 Jan;39(4):497–545.
221. Wood TR, Dawson CR. Bacteriologic studies of a trachomatous population. Am J Ophthalmol. 1967 May;63(5):Suppl:1298-301.
222. Nema H, Bal A, Nath K, Shukla B. Bacterial flora of the trachomatous conjunctiva. Br J Ophthalmol. 1964 Dec;48:690–1.
223. Woolridge R, Gillmore J. Bacteriological studies on Trachomatous and Normal Persons from Three Areas on Taiwan. Bull World Health Organ. 1962;26:789–95.
224. Martin DL, Bid R, Sandi F, Goodhew EB, Massae PA, Lasway A, et al. Serology for Trachoma Surveillance after Cessation of Mass Drug Administration. PLoS Negl Trop Dis. 2015;9(2):e0003555.
225. Mabey DC, Robertson JN, Ward ME. Detection of Chlamydia trachomatis by enzyme immunoassay in patients with trachoma. Lancet. 1987 Dec 26;2(8574):1491–2.
226. LeBar W, Herschman B, Jemal C, Pierzchala J. Comparison of DNA probe, monoclonal antibody enzyme immunoassay, and cell culture for the detection of Chlamydia trachomatis. J Clin Microbiol. 1989;27(5):826–8.
227. Solomon AW, Peeling RW, Foster A, Mabey DCW. Diagnosis and assessment of trachoma. Clin Microbiol Rev. 2004 Oct;17(4):982–1011.
228. Papp JR, Schachter J, Gaydos CA, Van Der Pol B. Recommendations for the Laboratory-Based Detection of Chlamydia trachomatis and Neisseria gonorrhoeae - 2014. Morb Mortal Wkly Rep Recomm reports. 2014 Mar 14;63(RR-02):1–19.
229. Dize L, West S, Quinn TC, Gaydos CA. Pooling ocular swab specimens from Tanzania for testing by Roche Amplicor and Aptima Combo 2 assays for the detection of Chlamydia trachomatis: accuracy and cost-savings. Diagn Microbiol Infect Dis. 2013 Dec;77(4):289–91.
230. Jenson A, Dize L, Mkocha H, Munoz B, Lee J, Gaydos C, et al. Field evaluation of the Cepheid GeneXpert Chlamydia trachomatis assay for detection of infection in a trachoma endemic community in Tanzania. PLoS Negl Trop Dis. 2013 Jan;7(7):e2265.
231. Roberts CH, Last A, Molina-Gonzalez S, Cassama E, Butcher R, Nabicassa M, et al. Development and Evaluation of a Next-Generation Digital PCR Diagnostic Assay for Ocular Chlamydia trachomatis Infections. J Clin Microbiol. 2013 Jul;51(7):2195–203.
232. Last AR, Burr SE, Alexander N, Harding-Esch EM, Roberts C h., Nabicassa M, et al. Spatial clustering of high load ocular Chlamydia trachomatis infection in trachoma: A cross-sectional population-based study. Pathog Dis. 2017;ftx050.
233. Keenan JD, See CW, Moncada J, Ayele B, Gebre T, Stoller NE, et al. Diagnostic characteristics of tests for ocular Chlamydia after mass azithromycin distributions. Vol. 53, Investigative Ophthalmology & Visual Science. 2012. p. 235–40.
234. Wang J, Zhang Y, Lu C, Lei L, Yu P, Zhong G. A genome-wide profiling of the humoral

immune response to Chlamydia trachomatis infection reveals vaccine candidate antigens expressed in humans. J Immunol. 2010 Aug 1;185(3):1670–80.

235. Sanchez-Campillo M, Bini L, Comanducci M, Raggiaschi R, Marzocchi B, Pallini V, et al. Identification of immunoreactive proteins of Chlamydia trachomatis by Western blot analysis of a two-dimensional electrophoresis map with patient sera. Electrophoresis. 1999 Aug;20(11):2269–79.
236. Eckert LO, Hawes SE, Wölner-Hanssen P, Money DM, Peeling RW, Brunham RC, et al. Prevalence and correlates of antibody to chlamydial heat shock protein in women attending sexually transmitted disease clinics and women with confirmed pelvic inflammatory disease. J Infect Dis. 1997;175(6):1453–8.
237. Peeling RW, Bailey RL, Conway DJ, Holland MJ, Campbell AE, Jallow O, et al. Antibody response to the 60-kDa chlamydial heat-shock protein is associated with scarring trachoma. J Infect Dis. 1998;177(1):256–9.
238. Kari L, Bakios LE, Goheen MM, Bess LN, Watkins HS, Southern TR, et al. Antibody signature of spontaneous clearance of Chlamydia trachomatis ocular infection and partial resistance against re-challenge in a nonhuman primate trachoma model. PLoS Negl Trop Dis. 2013;7(5):e2248.
239. GHAEM-MAGHAMI S, Ratti G, Ghaem-Maghami M, Comanducci M, Hay PE, Bailey RL, et al. Mucosal and systemic immune responses to plasmid protein pgp3 in patients with genital and ocular Chlamydia trachomatis infection. Clin Exp Immunol. Wiley-Blackwell; 2003 Jun;132(3):436–42.
240. Ghaem-Maghami S, Bailey RL, Mabey DC, Hay PE, Mahdi OS, Joof HM, et al. Characterization of B-cell responses to Chlamydia trachomatis antigens in humans with trachoma. Infect Immun. 1997 Dec;65(12):4958–64.
241. Comanducci M, Manetti R, Bini L, Santucci A, Pallini V, Cevenini R, et al. Humoral immune response to plasmid protein pgp3 in patients with Chlamydia trachomatis infection. Infect Immun. 1994;62(12):5491–7.
242. Wills GS, Horner PJ, Reynolds R, Johnson AM, Muir DA, Brown DW, et al. Pgp3 antibody enzyme-linked immunosorbent assay, a sensitive and specific assay for seroepidemiological analysis of Chlamydia trachomatis infection. Clin Vaccine Immunol. 2009 Jun;16(6):835–43.
243. Goodhew EB, Priest JW, Moss DM, Zhong G, Munoz B, Mkocha H, et al. CT694 and pgp3 as serological tools for monitoring trachoma programs. PLoS Negl Trop Dis. 2012 Jan;6(11):e1873.
244. Martin DL, Wiegand R, Goodhew B, Lammie P, Black CM, West S, et al. Serological Measures of Trachoma Transmission Intensity. Sci Rep. 2015;5:18532.
245. Goodhew EB, Morgan SM, Switzer AJ, Munoz B, Dize L, Gaydos C, et al. Longitudinal analysis of antibody responses to trachoma antigens before and after mass drug administration. BMC Infect Dis. 2014 Apr 22;14(1):216.
246. Horner PJ, Wills G, Reynolds R, Johnson A, Muir D, Winston A, et al. Effect of time since exposure to Chlamydia trachomatis on chlamydia antibody detection in women: a

cross-sectional study. Sex Transm Infect. 2013;89(5):398–403.

247. Zambrano A, Sharma S, Crowley K, Dize L, Muñoz B, Mishra S, et al. The World Health Organization Recommendations for Trachoma Surveillance, Experience in Nepal and Added Benefit of Testing for Antibodies to Chlamydia trachomatis pgp3 Protein: NESTS Study. PLoS Negl Trop Dis. 2016;10(9):e0005003.
248. West SK, Munoz B, Weaver J, Mrango Z, Dize L, Gaydos C, et al. Can We Use Antibodies to Chlamydia trachomatis as a Surveillance Tool for National Trachoma Control Programs? Results from a District Survey. PLoS Negl Trop Dis. 2016;10(1):e0004352.
249. Hu VH, Holland MJ, Burton MJ. Trachoma: protective and pathogenic ocular immune responses to Chlamydia trachomatis. PLoS Negl Trop Dis. 2013 Jan;7(2):e2020.
250. Rasmussen SJ, Eckmann L, Quayle AJ, Shen L, Zhang YX, Anderson DJ, et al. Secretion of proinflammatory cytokines by epithelial cells in response to Chlamydia infection suggests a central role for epithelial cells in chlamydial pathogenesis. J Clin Invest. 1997 Jan 1;99(1):77–87.
251. Natividad A, Freeman TC, Jeffries D, Burton MJ, Mabey DCW, Bailey RL, et al. Human conjunctival transcriptome analysis reveals the prominence of innate defense in Chlamydia trachomatis infection. Infect Immun. 2010 Nov;78(11):4895–911.
252. Abu el-Asrar AM, Geboes K, Tabbara KF, Al-Kharashi SA, Missotten L, Desmet V. Immunopathogenesis of conjunctival scarring in trachoma. Eye (Lond). 1998 Jan;12 (Pt 3a:453–60.
253. Reacher MH, Pe'er J, Rapoza PA, Whittum-Hudson JA, Taylor HR. T cells and trachoma. Their role in cicatricial disease. Ophthalmology. 1991 Mar;98(3):334–41.
254. Gall A, Horowitz A, Joof H, Natividad A, Tetteh K, Riley E, et al. Systemic effector and regulatory immune responses to chlamydial antigens in trachomatous trichiasis. Front Microbiol. 2011 Jan;2:10.
255. Burton MJ, Bailey RL, Jeffries D, Mabey DCW, Holland MJ. Cytokine and fibrogenic gene expression in the conjunctivas of subjects from a Gambian community where trachoma is endemic. Infect Immun. 2004 Dec;72(12):7352–6.
256. Burton MJ, Ramadhani A, Weiss HA, Hu V, Massae P, Burr SE, et al. Active trachoma is associated with increased conjunctival expression of IL17A and profibrotic cytokines. Infect Immun. 2011 Dec;79(12):4977–83.
257. Burton MJ, Rajak SN, Bauer J, Weiss HA, Tolbert SB, Shoo A, et al. Conjunctival transcriptome in scarring trachoma. Infect Immun. 2011 Jan;79(1):499–511.
258. Derrick T, Roberts C h, Rajasekhar M, Burr SE, Joof H, Makalo P, et al. Conjunctival MicroRNA expression in inflammatory trachomatous scarring. PLoS Negl Trop Dis. 2013 Jan;7(3):e2117.
259. Hu VH, Weiss HA, Ramadhani AM, Tolbert SB, Massae P, Mabey DCW, et al. Innate immune responses and modified extracellular matrix regulation characterize bacterial infection and cellular/connective tissue changes in scarring trachoma. Infect Immun. 2012 Jan;80(1):121–30.

260. Natividad A, Wilson J, Koch O, Holland MJ, Rockett K, Faal N, et al. Risk of trachomatous scarring and trichiasis in Gambians varies with SNP haplotypes at the interferon-gamma and interleukin-10 loci. Genes Immun. 2005 Jun;6(4):332–40.
261. Natividad A, Hanchard N, Holland MJ, Mahdi OSM, Diakite M, Rockett K, et al. Genetic variation at the TNF locus and the risk of severe sequelae of ocular Chlamydia trachomatis infection in Gambians. Genes Immun. 2007 Jun;8(4):288–95.
262. Natividad A, Cooke G, Holland MJ, Burton MJ, Joof HM, Rockett K, et al. A coding polymorphism in matrix metalloproteinase 9 reduces risk of scarring sequelae of ocular Chlamydia trachomatis infection. BMC Med Genet. 2006 Jan;7:40.
263. Roberts C, Molina S, Makalo P, Joof H, Harding-Esch EM, Burr SE, et al. Conjunctival Scarring in Trachoma Is Associated with the HLA-C Ligand of KIR and Is Exacerbated by Heterozygosity at KIR2DL2/KIR2DL3. PLoS Negl Trop Dis. 2014 Mar;8(3):e2744.
264. Roberts C h, Franklin CS, Makalo P, Joof H, Sarr I, Mahdi OS, et al. Conjunctival fibrosis and the innate barriers to Chlamydia trachomatis intracellular infection: a genome wide association study. Sci Rep. 2015 Jan;5:17447.
265. Meng N, Seiha D, Thorn P, Willis R, Flueckiger RM, Dejene M, et al. Assessment of Trachoma in Cambodia: Trachoma Is Not a Public Health Problem. Ophthalmic Epidemiol. 2016 Oct 11;1–5.
266. Cowling CS, Liu BC, Snelling TL, Ward JS, Kaldor JM, Wilson DP. National Trachoma Surveillance Annual Report, 2012. Commun Dis Intell. 2015;39(1):E146–57.
267. Polack S, Brooker S, Kuper H, Mariotti S, Mabey D, Foster A. Mapping the global distribution of trachoma. Bull World Health Organ. 2005 Dec;83(12):913–9.
268. United Nations. United Nations Development Programme (UNDP) Human development index. [Internet]. [cited 2014 Apr 27]. Available from: http://hdr.undp.org/en/data
269. Friedlaender JS, Friedlaender FR, Reed FA, Kidd KK, Kidd JR, Chambers GK, et al. The genetic structure of Pacific Islanders. PLoS Genet. 2008 Jan 18;4(1):e19.
270. Keeffe JE, Konyama K, Taylor HR. Vision impairment in the Pacific region. Br J Ophthalmol. England: BMJ Group; 2002 Jun;86(6):605–10.
271. Newland HS, Harris MF, Walland M, McKnight D, Galbraith JE, Iwasaki W, et al. Epidemiology of blindness and visual impairment in Vanuatu. Bull World Health Organ. 1992 Jan;70(3):369–72.
272. Newland HS, Woodward AJ, Taumoepeau LA, Karunaratne NS, Duguid IG. Epidemiology of blindness and visual impairment in the kingdom of Tonga. Br J Ophthalmol. 1994 May;78(5):344–8.
273. Heriot WJ, Crock GW, Taylor R, Zimmet P. Ophthalmic findings among one thousand inhabitants of Rarotonga, Cook Islands. Aust J Opthalmology. 1983 May;11(2):81–94.
274. Ramke J, Brian G, du Toit R. Eye disease and care at hospital clinics in Cook Islands, Fiji, Samoa and Tonga. Clin Experiment Ophthalmol. 2007 Jan;35(7):627–34.
275. Ramke J, Brian G, Maher L, Qoqonokana M, Szetu J. Prevalence and causes of blindness and low vision among adults in Fiji. Clin Exp Ophthalmol. 2012;40(5):490–496.
276. Lees J, McCool J, Woodward A. Eye health outreach services in the Pacific Islands

region: an updated profile. N Z Med J. New Zealand; 2015 Jan 21;128(1420):25–33.
277. QGIS Development Team. QGIS Geogrpahic Information System. 2016.
278. Macallan AF. Trachoma in the British Colonial Empire - its relation to blindness, the existing means of relief, means of prophylaxis. Br J Ophthalmol. 1934;18(11):625–45.
279. Ward B. The prevalence of active trachoma in Fiji. Am J Ophthalmol. 1965;59:458–63.
280. Swanston C. [Trachoma in the Fiji Islands]. Rev Int Trach. 1953 Jan;30(3):374–94.
281. Stuppel R. Trachoma in Fiji - an original investigation. Br J Ophthalmol. 1933 Feb;17(2):88–97.
282. Dethlefs R. The trachoma status and blindness rates of selected areas of Papua New Guinea in 1979-80. Aust J Ophthalmol. 1982 Feb;10(1):13–8.
283. Mann I. Some Islands. In: Climate, race, culture and eye disease. 1st ed. Springfield, IL, USA: Charles C Thomas; 1966.
284. Kline K, McCarthy JS, Pearson M, Loukas A, Hotez PJ. Neglected tropical diseases of Oceania: review of their prevalence, distribution, and opportunities for control. PLoS Negl Trop Dis. 2013 Jan;7(1):e1755.
285. Bodian M. Trachoma: a possible carrier state. Arch Ophthalmol. 1947;38(4):450–60.
286. Grayston J, Wang S, Woolridge R, Yang Y, Johnston P. Trachoma – studies of etiology, laboratory diagnosis and prevention. J Am Med Assoc. 1960;172(15):1577–1586.
287. International Agency for the Prevention of Blindness. Trachoma Mapping in the Pacific. Melbourne, Australia; 2013.
288. Ko R, Macleod C, Pahau D, Sokana O, Keys D, Burnett A, et al. Population-Based Trachoma Mapping in Six Evaluation Units of Papua New Guinea. Ophthalmic Epidemiol. Taylor & Francis; 2016;0(0):1–10.
289. Taleo F, Macleod CK, Marks M, Sokana O, Last AR, Willis R, et al. Integrated mapping of yaws and trachoma in the five northern-most provinces Vanuatu. PLoS Negl Trop Dis. 2017;11(1):e0005267.
290. Sokana O, Macleod CK, Jack K, Butcher R, Marks M, Willis R, et al. Mapping trachoma in The Solomon Islands – results from the Global Trachoma Mapping Project. Ophthalmic Epidemiol. 2016;23(Suppl. 1):15–21.
291. Kama M, Cama A, Rawalai K, Koroivueta J. Active Ocular Trachoma In Fiji- A Population Based Prevalence Survey. Fiji J Public Heal. 2013;2(2):11–7.
292. Mueller AJ. Assessment of the Prevalence of Trachoma on Kiritimati, Republic of Kiribati. 2016.
293. Solomon Island Government. Report on 2009 population and housing census. 2011.
294. Fiji Bureau of Statistics. Key Census Statistics. 2012.
295. Diisango S. Ministry launches war against trachoma. Solomon Star.
296. Vanuatu National Statistics Office. National Census of Population and Housing. 2009.
297. Kiribati National Statistics Office. Report on the Kiribati 2010 Cenus of Population and Housing. Bairiki: Ministry of Finance and Economic Planning, Kiribati; 2012.
298. Landis JR, Koch GG. The Measurement of Observer Agreement for Categorical Data. Biometrics. 1977 Mar;33(1):159.

299. Macleod CK, Butcher R, Mudaliar UU, Natutusau K, Pavluck AL, Willis R, et al. Low prevalence of ocular Chlamydia trachomatis infection and active trachoma in the Western Division of Fiji. PLoS Negl Trop Dis. 2016;10(7):e0004798.
300. Dize L, Gaydos CA, Quinn TC, West SK. Stability of Chlamydia trachomatis on storage of dry swabs for accurate detection by nucleic acid amplification tests. J Clin Microbiol. 2014 Dec 24;53(3):1046–7.
301. Gaydos CA, Farshy C, Barnes M, Quinn N, Agreda P, Rivers CA, et al. Can mailed swab samples be dry-shipped for the detection of Chlamydia trachomatis, Neisseria gonorrhoeae, and Trichomonas vaginalis by nucleic acid amplification tests ? Diagn Microbiol Infect Dis. Elsevier Inc.; 2012;73(1):16–20.
302. R Core Team. R: A Language and Environment for Statistical Computing [Internet]. R Foundation for Statistical Computing. 2014. Available from: http://www.r-project.org
303. van Dommelen L, Wolffs PFG, van Tiel FH, Dukers N, Herngreen SB, Bruggeman CA, et al. Influence of temperature, medium, and storage duration on Chlamydia trachomatis DNA detection by PCR. J Clin Microbiol. 2013 Mar;51(3):990–2.
304. Macleod C, Yalen C, Butcher R, Mudaliar U, Natutusau K, Rainima-Qaniuci M, et al. Eyelash epilation in the absence of trichiasis: results of a population-based prevalence survey in the Western Division of Fiji. PLoS Negl Trop Dis. 2017;11(1):e0005277.
305. Cocks N, Rainima-Qaniuci M, Yalen C, Macleod C, Nakolinivalu A, Migchelsen S, et al. Community seroprevalence survey for yaws and trachoma in the Western Division of Fiji. Trans R Soc Trop Med Hyg. England; 2016 Dec;110(10):582–7.
306. Harding-Esch EM, Edwards T, Mkocha H, Munoz B, Holland MJ, Burr SE, et al. Trachoma prevalence and associated risk factors in the gambia and Tanzania: baseline results of a cluster randomised controlled trial. PLoS Negl Trop Dis. 2010 Jan;4(11):e861.
307. Kirkwood B, Sterne JA. Calculation of required sample size. In: Essentail Medical Statistics. 2nd ed. Oxford, UK: Blackwell Publishing Ltd; 2003. p. 413–28.
308. Seth-Smith HMB, Harris SR, Scott P, Parmar S, Marsh P, Unemo M, et al. Generating whole bacterial genome sequences of low-abundance species from complex samples with IMS-MDA. Nat Protoc. 2013 Dec;8(12):2404–12.
309. Benaglia T, Chauveau D, Hunter DR, Young DS. mixtools: An R package for analyzing mixture models. J Stat Softw. 2009;32(1):1–29.
310. McLachlan G, Peel D. Finite Mixture Models. 1st ed. Hoboken, USA: John Wiley & Sons, Ltd; 2000.
311. Schachter J, Chow JM, Howard H, Bolan G, Moncada J. Detection of Chlamydia trachomatis by nucleic acid amplification testing: our evaluation suggests that CDC-recommended approaches for confirmatory testing are ill-advised. J Clin Microbiol. 2006 Jul;44(7):2512–7.
312. Porcella SF, Carlson JH, Sturdevant DE, Sturdevant GL, Kanakabandi K, Virtaneva K, et al. Transcriptional Profiling of Human Epithelial Cells Infected with Plasmid-Bearing and Plasmid-Deficient Chlamydia trachomatis. Infect Immun. 2015 Feb;83(2):534–43.

313. Burton MJ, Holland MJ, Jeffries D, Mabey DCW, Bailey RL. Conjunctival chlamydial 16S ribosomal RNA expression in trachoma: Is chlamydial metabolic activity required for disease to develop? Clin Infect Dis. 2006;42(4):463–70.
314. Meyler KL, Meehan M, Bennett D, Cunney R, Cafferkey M. Development of a diagnostic real-time polymerase chain reaction assay for the detection of invasive Haemophilus influenzae in clinical samples. Diagn Microbiol Infect Dis. 2012 Dec;74(4):356–62.
315. Greiner O, Day PJR, Altwegg M, Nadal D. Quantitative detection of Moraxella catarrhalis in nasopharyngeal secretions by real-time PCR. J Clin Microbiol. 2003 Apr;41(4):1386–90.
316. Carvalho M da GS, Tondella ML, McCaustland K, Weidlich L, McGee L, Mayer LW, et al. Evaluation and improvement of real-time PCR assays targeting lytA, ply, and psaA genes for detection of pneumococcal DNA. J Clin Microbiol. 2007 Aug 1;45(8):2460–6.
317. Okolie CE, Wooldridge KG, Turner DP, Cockayne A, James R. Development of a new pentaplex real-time PCR assay for the identification of poly-microbial specimens containing Staphylococcus aureus and other staphylococci, with simultaneous detection of staphylococcal virulence and methicillin resistance markers. Mol Cell Probes. 2015 Jun;29(3):144–50.
318. Yuan S, Cohen DB, Ravel J, Abdo Z, Forney LJ. Evaluation of methods for the extraction and purification of DNA from the human microbiome. PLoS One. Public Library of Science; 2012;7(3):e33865.
319. Willcox MDP. Characterization of the normal microbiota of the ocular surface. Exp Eye Res. 2013 Jun 22;
320. Varughese S, Gupta N. Ocular cicatricial pemphigoid: all conjunctival scarring is not trachoma. Indian J Ophthalmol. 2003;51(4):361.
321. Faraj HG, Hoang-Xuan T. Chronic cicatrizing conjunctivitis. Curr Opin Ophthalmol. 2001;12(4):250–7.
322. Marks M, Kako H, Butcher R, Lauri B, Puiahi E, Pitakaka R, et al. Prevalence of sexually transmitted infections in female clinic attendees in Honiara, Solomon Islands. BMJ Open. 2015;5(4).
323. Nijhuis RHT, Severs TT, Van der Vegt DSJM, Van Zwet AA, Kusters JG. High levels of macrolide resistance-associated mutations in Mycoplasma genitalium warrant antibiotic susceptibility-guided treatment. J Antimicrob Chemother. 2015;70(9):2515–2518.
324. Zhu B, Bu J, Li W, Zhang J, Huang G, Cao J, et al. High resistance to azithromycin in clinical samples from patients with sexually transmitted diseases in Guangxi Zhuang Autonomous Region, China. PLoS One. 2016;11(7):6–13.
325. Papp JR, Abrams AJ, Nash E, Katz AR, Kirkcaldy RD, Connor NPO, et al. Azithromycin Resistance and Decreased Ceftriaxone Susceptibility in Neisseria gonorrhoeae, Hawaii, USA. Emerg Infect Dis. 2017;23(5):25–7.
326. WHO. Global Health Sector Strategy on Sexually Transmitted Infections 2016-2021. World Heal Organ. 2016;(June).

10. APPENDICES

Appendix 1. Manuscript

Cocks N, Rainima-Qaniuci M, Yalen C, Macleod C, Nakolinivalu A, Migchelsen S, Roberts Ch, Butcher R, Kama M, Mabey D, Marks M. Community serorprevalence survey for yaws and trachoma in the Western Division of Fiji. *Trans R Soc Trop Med Hyg.* 2016; 1–6

The study in Appendix 2 was not led by the candidate and is not submitted as part of the thesis. The paper is included here as an appendix due to its relevance to the content of chapter four.

Appendix 2. Manuscript

Macleod C, Yalen C, Butcher R, Mudaliar U, Natutusau K, Rainima-Qaniuchi M, Haffenden C, Watson C, Cocks N, Cikamatana L, Roberts Ch, Marks M, Rafai E, Mabey D, Kama M, Solomon AW. Eyelash epilation in the absence of trichiasis: results of a population-based prevalence survey in the Western Division of Fiji. *PLoS NTDs.* 2017. 11(1): e0005277

The study in Appendix 3 was not led by the candidate and is not submitted as part of the thesis. The paper is included here as an appendix due to its relevance to the content of chapter four.

RESEARCH PAPER COVER SHEET

SECTION A – Student Details

Student	*Robert Butcher*
Principal Supervisor	*Chrissy h. Roberts*
Thesis Title	*Using alternate indicators of trachoma to estimate prevalence, characterise disease and define need for public health intervention: Evidence from the Pacific Islands*

If the Research Paper has previously been published, please complete Section B, if not please move to Section C

SECTION B – Paper already published

Where was the work published?	*Transactions of the Royal Society for Tropical Medicine and Hygiene*		
When was the work published?	*October 2016*		
If the work was published prior to registration for your research degree, give a brief rationale for its inclusion	*NA*		
Have you retained copyright for the work?*	*Yes*	**Was the work subject to academic peer review?**	*Yes*

If yes, please attach evidence of retention. If no, or if the work is being included in its published format, please attach evidence of permission from the copyright holder (publisher or other author) to include this work.* ***Work published under Creative Commons Attribution 4.0 International Open Access License.*

SECTION C – Prepared for publication, but not yet published

Where is the work intended to be published?	*NA*
Please list the paper's authors in the intended authorship order:	*NA*
Stage of publication	*NA*

SECTION D – Multi-authored work

For multi-authored work, give full details of your role in the research included in the paper and in the preparation of the paper.	*I obtained the funding for this study with Anthony Solomon and Chrissy Roberts. Michael Marks and Colin Macleod led the study. I helped to conceive the study with Michael and Colin. I planned the study, prepared and submitted the local ethics proposal with Michael, Colin and Mike Kama. Naomi Cocks and Chelsea Yalen conducted the field work. I supported Naomi and Chelsea in the field. I helped the lead authors to analyse the data, prepare and revise the manuscript.*

Student signature:  **Date:** 09/12/16

Supervisor signature:  **Date:** 19/12/16

Trans R Soc Trop Med Hyg 2016; 1–6
doi:10.1093/trstmh/trw069

Community seroprevalence survey for yaws and trachoma in the Western Division of Fiji

Naomi Cocks[a], Merelesita Rainima-Qaniuci[b], Chelsea Yalen[a], Colin Macleod[a], Apisalome Nakolinivalu[c], Stephanie Migchelsen[a], Chrissy h. Roberts[a], Robert Butcher[a], Mike Kama[d], David Mabey[a,e] and Michael Marks[a,e,*]

[a]Clinical Research Department, Faculty of Infectious and Tropical Diseases, London School of Hygiene & Tropical Medicine, Keppel Street, London WC1E 7HT, UK; [b]World Health Organization, Fiji Country Office, Fiji; [c]Department of Dermatology, Tamavua Twomey Hospital, Fiji; [d]Fiji Centre for Communicable Disease, Ministry of Health, Suva, Fiji; [e]Hospital for Tropical Diseases, University College London Hospitals NHS Trust, London WC1E 6JB, UK

*Corresponding author: Present address: London School of Hygiene & Tropical Medicine, Keppel Street, London WC1E 7HT, UK; E-mail: michael.marks@lshtm.ac.uk

Received 13 September 2016; revised 16 October 2016; editorial decision 19 October 2016

Introduction: Both yaws and trachoma are endemic in several countries in the Pacific. In co-endemic countries there may be potential synergies between both control programmes.

Methods: We undertook a cluster randomised trachoma and yaws seroprevalence survey of children in the Western Division of Fiji. Children were examined for skin lesions consistent with active yaws. A dried blood spot was collected which was tested using the *Treponema pallidum* particle agglutination (TPPA) test and an ELISA to detect antibodies against Pgp3.

Results: A total of 607 children from 305 households across 23 villages were recruited into the survey. On skin examination, no child had clinical evidence of yaws, and the TPPA assay was negative in all children (0%, 95% CI 0.0–0.6). The seroprevalence of Pgp3 antibodies was 20.9% (95% CI 17.8–24.6%).

Discussion: In this study there was neither clinical nor serological evidence that transmission of yaws was ongoing. The Pgp3 seroprevalence pattern was consistent with either low level transmission of ocular *Chlamydia trachomatis* or exposure to *C. trachomatis* in the birth canal which is consistent with a survey conducted in the same region in 2013. These data suggest neither yaws nor ocular chlamydia infection are a significant public health problem in the Western Division of Fiji.

Keywords: Fiji, Neglected tropical diseases, Scabies, Trachoma, Yaws

Introduction

Yaws, caused by *Treponema pallidum* subsp. *pertenue*, is endemic in several countries in the Pacific.[1] Trachoma, caused by ocular infection with *Chlamydia trachomatis*, is the leading infectious cause of blindness and is also endemic in the Pacific.[2,3] Both diseases are classified as neglected tropical diseases (NTDs) by WHO. WHO has targets for both the global eradication of yaws and elimination of blinding trachoma as a public health problem by 2020.[4,5] Central to the strategy for both of these targets is the use of community mass treatment with azithromycin, which is an effective therapy for both organisms. In countries where both yaws and trachoma are endemic there may be potential synergies between the control programmes at multiple levels including survey, intervention and surveillance.[6,7]

Of the countries where yaws remains endemic, three of the most high-burden countries are in the Pacific, including Papua New Guinea, the Solomon Islands and Vanuatu.[8] Yaws has previously been reported in many other countries in the Pacific, including Fiji, but there are no current data from Fiji on the prevalence of active disease or the seroprevalence of infection.[9] Accurate data on both clinical and serological prevalence of yaws in Fiji are needed to guide programmatic decision making.

Trachoma has been reported to be endemic in a number of countries in the Pacific, although population-based mapping studies have suggested a relatively low number of cases of trichiasis – the late, blinding, stage of the disease – in the

region. A recent survey completed in the Western Division of Fiji reported a low prevalence of ocular infection with *C. trachomatis* among children, and similar findings have been reported from other Pacific countries, including the Solomon Islands.[10]

Serology has recently emerged as a potential surveillance tool for trachoma programmes. Current measures of ocular infection (directly by PCR, or indirectly by clinical examination) provide only a cross-sectional snapshot of community prevalence, without necessarily giving any information about the changing exposure in a population over time. For trachoma, antibodies to Pgp3 are thought to be a long-lived and specific marker for prior *C. trachomatis* exposure.[11,12] Serology is also a major tool in surveillance for yaws. Treponemal antibodies persist for life and cannot distinguish between yaws and syphilis. A prevalence of treponemal antibodies greater than 1% amongst children has been suggested as a possible trigger for more detailed yaws mapping studies.[13]

The collection of dried blood spot samples in prevalence surveys have recently emerged as a practical tool to allow collection and storage of a large number of finger-prick blood samples from which the seroprevalence of several infections can be assessed. This has notable advantages in facilitating integrated NTD mapping and surveillance activities.[14,15] Prior to this study implementation of the SAFE strategy for elimination of trachoma had not yet commenced in Fiji and there had not been any azithromycin mass drug administration conducted in the country. We conducted an integrated survey to assess the seroprevalence of antibodies to *T. pallidum* and *C. trachomatis* in order to evaluate the need for further interventions for these NTDs in Fiji.

Methods

We conducted a population-based cluster-randomised survey in the Western Division of Fiji in July and August 2015, where we had previously documented a low prevalence of both clinical signs of active and ocular infection with *C. trachomatis*.[2] No mass distribution of azithromycin for trachoma or yaws has previously been carried out in this region, nor any recent penicillin-based mass treatment campaigns for yaws, to the best of the authors' knowledge. Data are reported in line with the STROBE guidelines for cross-sectional surveys (Supplementary File 1). Study data are available in Supplementary File 2.

Survey methodology

This was a two-stage cluster-randomised survey. Each cluster consisted of a single village. Villages were selected randomly, using probability proportional to size sampling, from a list of all communities in the Western Division of Fiji. On the day on the survey, in collaboration with local leaders, a list of all households was enumerated and 30 households selected using simple random sampling. All children aged 1–14 at sampled households were eligible for inclusion.

Data collection

A nurse was trained in standardised dermatology. Examinations included the head and neck, limbs and trunk but excluded the genitals and buttocks. Individual-level data were collected on age, gender, and the presence or absence of skin lesions. For each skin lesion we recorded the location, clinical appearance and whether the lesion was consistent with yaws or an alternative diagnosis. A clinical case of yaws was defined on the basis of typical clinical findings of papillomatous or chronic, painless ulcerative skin lesions and using the WHO pictorial guide to yaws.[16] The clinical diagnosis of scabies was based on features including morphology (burrows, papules, nodules, vesicles) and body distribution of rash; presence of pruritus on history or evidence of excoriation; contact history with individuals with a similar rash and itch; and consideration of differential diagnoses. The distribution of scabies lesions was noted using nine pre-defined body regions. Scabies severity was classified by the number of lesions present as mild (≤10 lesions), moderate (11–49 lesions) or severe (≥50 lesions or crusted scabies).

Active impetigo was diagnosed on the basis of discrete papular, pustular or ulcerative lesions with associated erythema, crusting, bullae or frank pus. Inactive impetigo was diagnosed by the presence of discrete, non-confluent healed superficial skin lesions. Severity of active impetigo was classified as very mild (≤5 lesions), mild (6–10 lesions), moderate (11–49 lesions) or severe (≥50 lesions).

All data were entered directly into Android smartphones using the OpenDataKit software package.[17] Individuals with a skin condition requiring treatment were referred to the local health clinic where they were treated free of charge in line with Ministry of Health guidelines.

A finger-prick blood sample was collected onto a filter paper from all children regardless of clinical features. Filter papers were air-dried and stored in a sealed bag with a desiccant sachet. In individuals with ulcerative or papillomatous skin lesions consistent with yaws we also collected a swab sample of lesion exudate. Exudate was transferred to a FTA Elute Micro Card (GE Healthcare, Chalfont St Giles, UK) using three firm side-to-side motions of the swab across the card. Each card was placed in its own re-sealable plastic packet with an individual desiccant sachet. All samples were transferred to Lautoka Hospital and frozen at −20°C and then shipped to the London School of Hygiene & Tropical Medicine (LSHTM), London, UK for testing.

Laboratory testing

Blood samples were tested at LSHTM. All laboratory testing was performed by individuals masked to the clinical findings. For yaws, a single extension of each filter paper sample was eluted as previously described and the elute tested using the *T. pallidum* particle agglutination test (TPPA; Mast Diagnostics, Bootle, UK).[18] For trachoma, a separate extension of each filter paper was tested using an ELISA for Pgp3 developed by the US Centres for Disease Control (Diana Martin, personal communication). Dried blood spots were eluted into 250 µL of 1× phosphate-buffered saline (PBS) with 2.5% weight-for-volume milk powder plus 0.3% volume-for-volume tween 20 (PBST-milk). Immunolon 2HB plates were coated overnight with 50 ng per well glutathione S-transferase

(GST)-tagged Pgp3 then blocked for 1 hour with PBST. Plates were bound for 2 hours with 50 µL of the elution mixture, then incubated at room temperature with rabbit anti-IgG for 1 hour. Plates were then incubated in the dark for 10 minutes with tetramethylbenzidine (TMB) and the absorbance read at 450 nm. Plates were washed four times with PBST between steps. A series of positive serum diluted in negative serum was run on each plate. Plates were re-run if the mean of each point in the dilution series fell more than 20% outside of pre-determined normal range. All specimens on the plate were normalised to a 20% dilution of presumed-positive material as that typically gave optical density (OD) readings around 1.

Statistical analysis

We calculated the clinical prevalence of yaws and other common skin infections. A positive cut-off value for the Pgp3 ELISA was defined using a mixtures model.[19] For the purposes of analysis we grouped age into 1–5 years, 6–10 years and 11–14 years of age. We categorised ethnicity as iTaukei, Indo-Fijian (the predominant ethnic groups in Fiji) or other. We calculated the seroprevalence of antibodies against *T. pallidum* and *C. trachomatis*. Random effects logistic regression was used to assess associations between age, sex, ethnicity and gender with both clinical findings and seroprevalence data controlling for the effect of clustering at household and village-level. Analysis was conducted in STATA 13.1 (StataCorp LP, College Station, TX, USA) and R 3.2.3 (the R Project for Statistical Computing).[20]

Sample size

We calculated that a sample size of 1005 children was needed to detect a seroprevalence of both treponemal antibodies and Pgp3 antibodies of 5% in 1–14 year-olds with a degree of precision of ±2%, a design effect of 2, and a 10% non-response rate. Based on the Fiji national census we calculated that 30 households in each of 30 villages would be sufficient to reach this sample size.

Ethical approval

Written informed consent was obtained from each participating child's parent or guardian by a member of staff fluent in the local dialect. Assent was obtained from all children with a signature or thumb print. Ethical approval for the study was granted by the ethics committees of the Fiji Ministry of Health (2015.65.WES) and LSHTM (10359).

Results

A total of 607 children from 305 households across 23 villages were recruited into the survey. The median age was 6 years (IQR 4–9), 312 children were male (51.4%). There were 493 individuals of iTaukei descent (81.2%), 91 individuals were Indo-Fijian (15.0%) and 23 children were of another ethnicity (3.8%).

On examination 184 children (30.3%) had at least one skin lesion. No skin lesions were considered to be clinically consistent with yaws. The most common skin lesions noted were scabies without secondary bacterial infection (n=59, 9.7%), scabies with bacterial skin infection (n=31, 5.1%) and impetigo without underlying scabies (n=24, 3.9%). Scabies was more common amongst children of iTaukei ethnicity (OR 2.9, p=0.015) and was the major risk factor for the presence of impetigo (OR 10.30, p<0.0001) with a population attributable fraction of 51.2%. Eczema (n=18, 2.9%), tinea corporis (n=16, 2.6%) and molluscum contagiosum (n=14, 2.3%) were also common (Figure 1).

Dried blood spots were collected from 593 children (96.7%). The TPPA was negative in all children enrolled in this study (95% CI 0–0.6%). Because no yaws-like lesions were identified no swabs for PCR were collected. Using the mixtures model an OD threshold of 0.72 was defined for the Pgp3 assay. Based on this cut-off the overall seroprevalence of Pgp3 antibodies was 20.9%. Seroprevalence increased with age from 15% in children aged 1 year to 22% amongst children aged 14 years. In univariate analysis both age and ethnicity were significantly associated with a positive Pgp3 ELISA (p=0.043 and p<0.001 respectively) but there was no association with gender (p=0.71). In multivariable analysis iTaukei ethnicity was associated with a significantly increased risk of Pgp3 seropositivity (aOR 9.82, 95% CI 2.94–32.79, p<0.001) (Table 1 and Figure 2).

Discussion

We found no evidence that yaws was still endemic in the Western Division of Fiji. No children were identified with clinical evidence of yaws and treponemal antibody testing was negative in all children. As these tests reflect lifetime exposure to *T. pallidum* this represents strong evidence that transmission of yaws is not ongoing in the communities where this study took place. Approximately one-fifth of children in the study had evidence of exposure to *C. trachomatis*. In a previous study in the Western Division of Fiji we reported a trachomatous inflammation – follicular (TF) prevalence of 2.8% and an ocular chlamydia infection prevalence of 2.3%.[2] These serology data are comparable to other settings where there is a low prevalence of both TF and ocular chlamydia infection.[21] Steeply increasing age-specific anti-pgp3 prevalence in children aged between 1 and 10 years is thought to be indicative of intense transmission of ocular

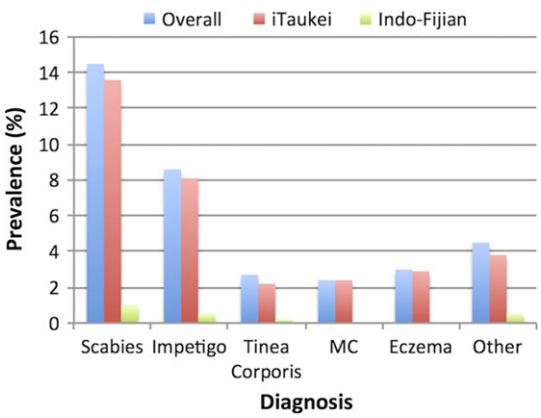


Figure 1. Clinical prevalence of common skin infections.

Table 1. Factors associated with Pgp3 antibody positivity

Variable	Indicative prevalence data	Adjusted odds ratio[a] (95% CI)	p-value[b]
Age (years)			
1–5 (n=267)	16.9%	1	p=0.0133
6–10 (n=218)	23.3%	1.83 (1.10–3.02)	
11–14 (n=99)	25.2%	2.20 (1.17–4.15)	
Ethnicity			
Indo-Fijian (n=86)	3.5%	1	p<0.0001
iTaukei (n=476)	24.8%	10.88 (3.12–37.92)	
Other (n=22)	9.1%	2.89 (0.39–21.05)	
Gender			
Male (n=308)	20.5%	1	NS
Female (n=276)	21.7%	0.89 (0.57–1.38)	

NS: not significant.
[a]All odds ratios are adjusted for age, gender and ethnicity.
[b]Likelihood ratio test.

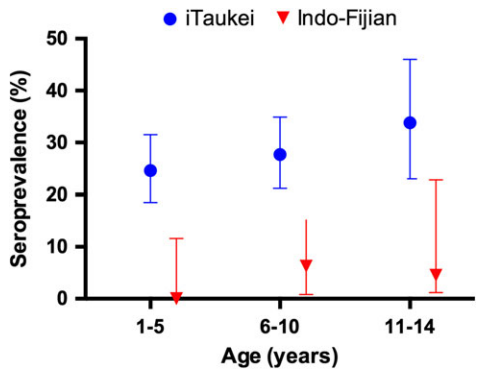


Figure 2. Seroprevalence of Pgp3 antibodies by age and ethnicity. Data are seroprevalence and 95% CIs. Only data for iTaukei and Indo-Fijian children are shown due to the small number of children of other ethnicities.

C. trachomatis; in this population the prevalence of anti-pgp3 antibodies was already high in the 1-year age group, suggesting the majority of seropositivity was acquired at in the first year of life.[12,21] As Pgp3 is conserved between urogenital and ocular strains of *C. trachomatis* we cannot discount the possibility that the Pgp3 seroprevalence may reflect transmission in the birth canal as well as, or indeed more than, ocular transmission of chlamydia. The prevalence of urogenital *C. trachomatis* infection amongst pregnant women has previously been reported to be high in Fiji and community prevalence surveys in other Pacific countries have also reported high rates of urogenital *C. trachomatis*.[22,23] In a study of pregnant women in Suva, Fiji, native Fijian women were found to have higher rates of urogenital infections, including *C. trachomatis,* than Indo-Fijian women.[24] Based on the current data it is unclear whether ocular *C. trachomatis* infection or transmission in the birth canal is the major driver of Pgp3 seropositivity in these communities. These data highlight the potential challenges of using Pgp3 as a sero-marker for trachoma in communities where the prevalence of genital chlamydia infection is also high.

Skin problems were common in this study population with scabies and impetigo the most commonly identified conditions. These findings are consistent with a national scabies prevalence survey that was previously conducted in Fiji.[25] As previously noted scabies was more common amongst iTaukei than indo-Fijians and was the major risk factor for impetigo.[25] The absence of both chronic ulcerative lesions and papillomatous lesions consistent with yaws is supported by our serological data showing an absence of treponemal antibody positivity. Anecdotally, health care staff in the Western Division of Fiji reported that they did not see children with skin lesions consistent with yaws presenting to health care clinics.

Our study has a number of limitations. Whilst survey clusters were chosen in advance, we did not achieve our desired sample size because of logistical difficulties on the days of surveys of particular communities and the time limitations of the survey team. This may have limited our ability to detect rare cases of yaws amongst these communities. This is of particular importance as yaws is known to be highly focal even in regions where it is endemic. However the absence of any positive treponemal antibody tests in children enrolled in this study provides strong evidence that transmission is not ongoing in the communities included in this study. The appropriate methodology for confirming yaws elimination has not yet been agreed but it is likely that large-scale seroprevalence surveys will be required. The reduced sample size also reduced the statistical power to assess associations between demographic variables and Pgp3 antibody status. Secondly, the study was conducted in only a limited geographic region of Fiji. Further studies in other parts of the country should be undertaken. Finally we did not collect linked clinical data on active trachoma or ocular swabs for PCR during this survey. We had conducted a previous survey in this region of Fiji two years earlier where we have demonstrated a low prevalence of TF (2.8%) and ocular *C. trachomatis* infection

(2.3%) amongst children[2] and where no cases of trachomatous trichasis were seen.

There is a need for better data to inform programmatic decision making and integrated approaches to NTDs in the Pacific. Our study has confirmed that yaws and trachoma mapping activities can be integrated and that it may be possible to expand this to other NTDs such as scabies. There is also scope for cooperation between mass treatment programs, for example co-administration of ivermectin, albendazole and azithromycin, where more than one treatment is indicated and appropriate safety and efficacy data exist.[26] Our data add to existing data suggesting that yaws is no longer a public health problem in Fiji and that trachoma is not likely to be one of the major causes of blindness in Fiji. Given the mixed findings of trachoma surveys conducted in Fiji early impact assessments should be conducted to guide Ministry of Health trachoma elimination activities.

Supplementary data

Supplementary data are available at Transactions online (http://trstmh.oxfordjournals.org/).

Authors' contributions: CM, MM, ChR, RB, MK, DM, MM designed the study. NC, MEQ, CY, AN, SM performed the study fieldwork and lab work. CM, MM, RB analysed the data and wrote the first draft of the manuscript. NC, MRQ, CY, AN, SM, ChR, MK and DM revised the manuscript. All authors read and approved the final manuscript. MM is the guarantor of the paper.

Acknowledgements: The authors are grateful to Dr Diana Martin (US Centres for Disease Control, Atlanta GA) for the provision of reagents and protocol for the anti-Pgp3 ELISA. The authors are grateful to Dr Anasaini Cama (International Association for the Prevention of Blindness, Western Pacific Region) for logistical support during the survey preparation.

Funding: CY and NC received an award for travel to the fieldwork site from the Trust Funds of the London School of Hygiene & Tropical Medicine. RB was supported by the Wellcome Trust [098521/B/12/Z]. CHR is supported by the Wellcome Trust Institutional Support Fund [105609/Z/14/Z]. MM is supported by the Wellcome Trust [102807/Z/13/Z]. The fieldwork and laboratory analyses were funded by the Fred Hollows Foundation Australia [FHF 1041]. None of the funders had any role in project design, in project implementation or analysis or interpretation of data, in the decisions on where, how or when to publish in the peer reviewed press, or in preparation of the manuscript.

Competing interests: None declared.

Ethical approval: Ethical approval for the study was granted by the ethics committees of the Fiji Ministry of Health (2015.65.WES) and LSHTM (10359).

References

1 Marks M, Solomon AW, Mabey DC. Endemic treponemal diseases. Trans R Soc Trop Med Hyg 2014;108:601–7.

2 Macleod CK, Butcher R, Mudaliar U et al. Low prevalence of ocular *Chlamydia trachomatis* infection and active trachoma in the Western Division of Fiji. PLoS Negl Trop Dis 2016;10:e0004798.

3 Sokana O, Macleod C, Jack K et al. Mapping trachoma in The Solomon Islands – results from the Global Trachoma Mapping Project. Ophthalmic Epidemiol 2016;Forthcoming.

4 Emerson PM, Burton MJ, Solomon AW et al. The SAFE strategy for trachoma control: using operational research for policy, and implementation. Bull World Health Organ 2006;84:613–9.

5 WHO. Eradication of yaws – the Morges Strategy. Wkly Epidemiol Rec 2012;87:189–94.

6 Marks M, Vahi V, Sokana O et al. Impact of community mass treatment with azithromycin for trachoma elimination on the prevalence of yaws. PLoS Negl Trop Dis 2015;9:e0003988.

7 Solomon AW, Marks M, Martin DL et al. Trachoma and yaws: common ground? PLoS Negl Trop Dis 2015;9:e0004071.

8 Mitjà O, Marks M, Konan DJP et al. Global epidemiology of yaws: a systematic review. Lancet Glob Health 2015;3:e324–31.

9 WHO. Global Health Observatory Data Repository. Status of endemicity for yaws. Data by country. Geneva: World Health Organization; 2014. http://apps.who.int/gho/data/node.main.NTDYAWSEND?lang=en [accessed 2 October 2016].

10 Butcher RMR, Sokana O, Jack K et al. Low prevalence of conjunctival infection with *Chlamydia trachomatis* in a treatment-naïve trachoma-endemic region of the Solomon Islands. PLoS Negl Trop Dis 2016;10:e0005051.

11 Goodhew EB, Priest JW, Moss DM et al. CT694 and pgp3 as serological tools for monitoring trachoma programs. PLoS Negl Trop Dis 2012;6:e1873.

12 Martin DL, Bid R, Sandi F et al. Serology for trachoma surveillance after cessation of mass drug administration. PLoS Negl Trop Dis 2015;9:e0003555.

13 WHO. Yaws strategy development: report of a meeting, 27–28 October 2014, Atlanta, GA, USA. Geneva: World Health Organization; 2015. http://apps.who.int/iris/handle/10665/170990 [accessed 8 October 2016].

14 Solomon AW, Engels D, Bailey RL et al. A diagnostics platform for the integrated mapping, monitoring, and surveillance of neglected tropical diseases: rationale and target product profiles. PLoS Negl Trop Dis 2012;6:e1746.

15 Lammie PJ, Moss DM, Goodhew EB et al. Development of a new platform for neglected tropical disease surveillance. Int J Parasitol 2012;42:797–800.

16 WHO. Yaws: recognition booklet for communities. Geneva: World Health Organization; 2012. http://apps.who.int/iris/bitstream/10665/75360/1/9789241504096_eng.pdf?ua=1 [accessed 8 October 2016].

17 Pavluck A, Chu B, Mann Flueckiger R, Ottesen E. Electronic data capture tools for global health programs: evolution of LINKS, an Android-, web-based system. PLoS Negl Trop Dis 2014;8:e2654.

18 Smit PW, Vlis T, van der Mabey D et al. The development and validation of dried blood spots for external quality assurance of syphilis serology. BMC Infect Dis 2013;13:102.

19 Parker RA, Erdman DD, Anderson LJ. Use of mixture models in determining laboratory criterion for identification of seropositive individuals: application to parvovirus B19 serology. J Virol Methods 1990;27:135–44.

20 Johnson P. adaptivetau: Tau-leaping stochastic simulation. R package version 2.2. 2014 https://CRAN.R-project.org/package=adaptivetau [accessed 8 October 2016].

21 Martin DL, Wiegand R, Goodhew B et al. Serological measures of trachoma transmission intensity. Sci Rep 2015;5:18532. doi:10.1038/srep18532.

22 Cliffe SJ, Tabrizi S, Sullivan EA, Pacific Islands Second Generation HIV Surveillance Group. Chlamydia in the Pacific region, the silent epidemic. Sex Transm Dis 2008;35:801–6.

23 Walsh MS, Hope E, Isaia L et al. Prevalence of *Chlamydia trachomatis* infection in Samoan women aged 18 to 29 and assessment of possible risk factors: a community-based study. Trans R Soc Trop Med Hyg 2015;109:245–51.

24 Gyaneshwar R, Nsanze H, Singh KP et al. The prevalence of sexually transmitted disease agents in pregnant women in Suva. Aust N Z J Obstet Gynaecol 1987;27:213–5.

25 Romani L, Koroivueta J, Steer AC et al. Scabies and impetigo prevalence and risk factors in Fiji: a national survey. PLoS Negl Trop Dis 2015;9:e0003452.

26 Coulibaly YI, Dicko I, Keita M et al. A cluster randomized study of the safety of integrated treatment of trachoma and lymphatic filariasis in children and adults in Sikasso, Mali. PLoS Negl Trop Dis 2013;7:e2221.

RESEARCH PAPER COVER SHEET

SECTION A – Student Details

Student	*Robert Butcher*
Principal Supervisor	*Chrissy h. Roberts*
Thesis Title	*Using alternate indicators of trachoma to estimate prevalence, characterise disease and define need for public health intervention: Evidence from the Pacific Islands*

If the Research Paper has previously been published, please complete Section B, if not please move to Section C

SECTION B – Paper already published

Where was the work published?	*NA*		
When was the work published?	*NA*		
If the work was published prior to registration for your research degree, give a brief rationale for its inclusion	*NA*		
Have you retained copyright for the work?*	*NA*	**Was the work subject to academic peer review?**	*NA*

If yes, please attach evidence of retention. If no, or if the work is being included in its published format, please attach evidence of permission from the copyright holder (publisher or other author) to include this work. ***Work published under Creative Commons Attribution 4.0 International Open Access License.*

SECTION C – Prepared for publication, but not yet published

Where is the work intended to be published?	*PLoS NTDs*
Please list the paper's authors in the intended authorship order:	*Macleod C, Yalen C, Butcher R, Mudaliar U, Natutusau K, Rainima-Qaniuchi M, Haffenden C, Watson C, Cocks N, Cikamatana L, Roberts Ch, Marks M, Rafai E, Mabey D, Kama M, Solomon AW.*
Stage of publication	*In press*

SECTION D – Multi-authored work

For multi-authored work, give full details of your role in the research included in the paper and in the preparation of the paper.	*I obtained the funding for this study with Anthony Solomon and Chrissy Roberts. Michael Marks and Colin Macleod led the study. I helped Colin to analyse the data from the focus group. I conceived, designed and planned the population-based study with Anthony Solomon, Michael, Mike Kama and Colin. Colin and Chelsea wrote the interview questions. Naomi Cocks and Chelsea Yalen conducted the field work. I helped Michael and Colin to prepare and submit the local ethics proposal. I supported Naomi and Chelsea in the field. I helped the lead authors to prepare and revise the manuscript.*

Student signature: _____ **Date:** 09/12/16

Supervisor signature: _____ **Date:** 19/12/16

PLOS | NEGLECTED TROPICAL DISEASES

RESEARCH ARTICLE

Eyelash Epilation in the Absence of Trichiasis: Results of a Population-Based Prevalence Survey in the Western Division of Fiji

Colin Macleod[1☯]*, Chelsea Yalen[1☯], Robert Butcher[1], Umesh Mudaliar[2], Kinisimere Natutusau[2], Mere Rainima-Qaniuci[3], Chris Haffenden[4], Conall Watson[5], Naomi Cocks[1], Luisa Cikamatana[2,6], Chrissy H. Roberts[1], Michael Marks[1,7], Eric Rafai[6], David C. W. Mabey[1], Mike Kama[6], Anthony W. Solomon[1,7]

1 Clinical Research Department, London School of Hygiene & Tropical Medicine, London, United Kingdom, **2** Ophthalmology Department, Lautoka Hospital, Lautoka, Fiji, **3** World Health Organisation, Fiji Country Office, Suva, Fiji, **4** Department of the History of Science and Ideas, Uppsala University, Uppsala, Sweden, **5** Department of Infectious Disease Epidemiology, London School of Hygiene & Tropical Medicine, London, United Kingdom, **6** Department of Communicable Diseases, Ministry of Health, Suva, Fiji, **7** Hospital for Tropical Diseases, London, United Kingdom

☯ These authors contributed equally to this work.
* colin.macleod@lshtm.ac.uk

OPEN ACCESS

Citation: Macleod C, Yalen C, Butcher R, Mudaliar U, Natutusau K, Rainima-Qaniuci M, et al. (2017) Eyelash Epilation in the Absence of Trichiasis: Results of a Population-Based Prevalence Survey in the Western Division of Fiji. PLoS Negl Trop Dis 11(1): e0005277. doi:10.1371/journal.pntd.0005277

Editor: Christian Johnson, Fondation Raoul Follereau, FRANCE

Received: July 15, 2016

Accepted: December 21, 2016

Published: January 23, 2017

Data Availability Statement: The linked demographic, GPS and clinical data are subject to a data-sharing agreement mandated by the London School of Hygiene & Tropical Medicine Ethics Review Board and signed by the Fiji Ministry of Health and LSHTM. Making these data available without restriction would breach the terms of that agreement, therefore, data can be accessed through contacting the National Health Research Ethics Committee of Fiji. Researchers can apply to

Abstract

Background

The WHO definition of trachomatous trichiasis (TT) is "at least one eyelash touching the globe, or evidence of recent epilation of in-turned eyelashes", reflecting the fact that epilation is used as a self-management tool for TT. In Fiji's Western Division, a high TT prevalence (8.7% in those aged ≥15 years) was reported in a 2012 survey, yet a 2013 survey found no TT and Fijian ophthalmologists rarely see TT cases. Local anecdote suggests that eyelash epilation is a common behaviour, even in the absence of trichiasis. Epilators may have been identified as TT cases in previous surveys.

Methods

We used a preliminary focus group to design an interview questionnaire, and subsequently conducted a population-based prevalence survey to estimate the prevalence of epilation in the absence of trichiasis, and factors associated with this behaviour, in the Western Division of Fiji.

Results

We sampled 695 individuals aged ≥15 years from a total of 457 households in 23 villages. 125 participants (18%) reported epilating their eyelashes at least once within the past year. Photographs were obtained of the eyes of 121/125 (97%) individuals who epilated, and subsequent analysis by an experienced trachoma grader found no cases of trachomatous conjunctival scarring or trichiasis. The age- and sex- adjusted prevalence of epilation in those

obtain access to the data by contacting the data manager at rwillis@taskforce.org.

Funding: CM, CY and NC received an award for travel to the fieldwork site from the Trust Funds of the London School of Hygiene & Tropical Medicine. RB was supported by the Wellcome Trust (098521/B/12/Z). MM is supported by the Wellcome Trust (102807/Z/13/Z). CHR is supported by a Wellcome Trust Institutional Support Fund (105609/Z/14/Z). AWS was a Wellcome Trust Intermediate Clinical Fellow (098521) at the London School of Hygiene & Tropical Medicine. The fieldwork and trachoma laboratory analyses were funded by the Fred Hollows Foundation Australia (FHF 1041). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.

aged ≥15 years was 8.6% (95% CI 5.7–11.3%). iTaukei ethnicity, female gender, and a higher frequency of drinking kava root were independently associated with epilation.

Conclusion

Epilation occurs in this population in the absence of trichiasis, with sufficient frequency to have markedly inflated previous estimates of local TT prevalence. Individuals with epilated eyelashes should be confirmed as having epilated in-turned eyelashes in an eye with scarring of the conjunctiva before being counted as cases of TT.

Author Summary

Trachoma, caused by infection with ocular strains of *Chlamydia trachomatis*, represents a major public health issue, and is targeted for global elimination as a public health problem by the year 2020. Until recently, data on trachoma in the Pacific Island states have been sparse, with marked variability in the findings of surveys. The most recent studies in Fiji's Western Division were conflicted in their estimates of the prevalence of the advanced, blinding, stage of the disease known as trachomatous trichiasis or "TT". TT results from repeated bouts of infection and resolution, leading to scarring of the eyelid tissue, which causes in-turning of the eyelashes in some individuals so that they grow to touch the globe of the eye. In populations without ready access to healthcare services, individuals may try to self-manage TT by epilating their eyelashes, so that the classical trichiasis sign of contact between eyelashes and eyeball is not seen in surveys. Therefore, the World Health Organization (WHO) definition of TT includes "evidence of recent epilation of in-turned eyelashes". In the Western Division of Fiji, we carried out a population-based prevalence survey to estimate the prevalence of this behaviour, and to examine associated risk factors. The estimated population prevalence of epilation was 8.6% of those aged ≥15 years, consistent with previously reported estimates of TT in this population, and, importantly, was not associated with any other evidence of advanced trachoma. These data suggest that eyelash epilation is common here, and could inflate estimates of TT wherever such a custom is common. In trachoma surveys, trachomatous scarring should be confirmed to be present when reporting the presence of TT.

Introduction

Trachoma is the leading infectious cause of blindness globally, responsible for the irreversible loss of vision in 1.9 million people.[1] It is caused by repeated bouts of conjunctival *Chlamydia trachomatis* infection and resolution during childhood, resulting in the gradual accumulation of trachomatous conjunctival scarring (TS). Scarring may progress to distortion of the eyelid and in-turning of the eyelashes to the point that they touch the eyeball (trachomatous trichiasis, TT). Abrasion of the cornea can lead to opacity and blindness[2].

There is international commitment to the global elimination of trachoma as a public health problem by 2020, defined as a reduction in the prevalence of TT unknown to the health system in adults aged ≥15 years to <0.2%, and a reduction in the prevalence of the active trachoma sign trachomatous inflammation—follicular in 1–9 year-olds to <5% [3], by means of the SAFE strategy[4]: Surgery for TT, Antibiotics to clear infection, Facial cleanliness and

Environmental improvement to reduce transmission[5]. Accurate estimates of the prevalence of TT are crucial for intervention planning and monitoring progress towards elimination.

TT is an irritating, painful condition that causes significant morbidity to affected individuals.[6,7] Those afflicted may find relief by epilation, which is a traditional treatment for TT in some settings. Epilation can lead to reasonable outcomes where surgery is unavailable, delayed or refused, particularly if caregivers are trained and equipped to do it well[8,9]. To acknowledge that this practice occurs and that if not recognised can obscure the presence of TT, the definition of TT in the WHO simplified trachoma grading system is "at least one eyelash rubs on the eyeball, or evidence of recent removal of in-turned eyelashes"[10].

The extent to which eyelash epilation in the absence of trichiasis can affect TT estimates in trachoma surveys is previously unstudied. Due to the low TT prevalence threshold required to declare elimination of trachoma, significant sources of false-positive TT diagnoses need to be identified. A small number of false positive TT cases recorded by survey teams, when extrapolated to population-level estimates, could lead to the unnecessary training of many trichiasis surgeons. This would be an unnecessary expense and needlessly divert trained healthcare personnel from their regular duties, in places where healthcare staff in general are often in short supply.

Fiji is an archipelago of over 300 islands in the South Pacific, divided geographically into four administrative divisions: Central, Northern, Eastern and Western, with a combined population of approximately 837,300 people.[11] Recent surveys have indicated that trachoma is endemic in Fiji, although prevalence estimates of TT have varied widely. A trachoma rapid assessment in 2007 found 59/313 (19%) of adults over the age of 40 years living in suspected high-risk areas to have evidence of TS, but did not find any cases of TT[12]. A population-based prevalence survey conducted in 2012 identified almost 150 people with TT, and estimated the population prevalence in the Western Division to be 8.7%, among the highest in the world[13]. In response to these results, a second population-based prevalence survey, supported by the Global Trachoma Mapping Project, was carried out in Fiji's Western Division in 2013 in order to re-estimate the prevalence of signs of trachoma and of conjunctival *C. trachomatis* infection. It found no cases of trichiasis in a study population of 2306 people in 31 communities: an estimated prevalence of trichiasis of 0%[14]. During that study, we heard local anecdotes that suggested some iTaukei Fijians may practice epilation in the absence of TT symptoms, as a sociocultural behaviour. These individuals might be incorrectly graded as having TT: an individual cannot truly have trichiasis if the epilated eyelashes are not in-turned. This could have major implications for trichiasis estimates and surgery planning in suspected trachoma-endemic populations in which such behaviour is common.

We sought to understand the motivations for and significance of eyelash epilation in Western Division by first convening a focus group of individuals who reported regularly practising this behaviour. Using their responses to design a questionnaire, we conducted a population-based prevalence survey to estimate the prevalence of epilation, and factors associated with it, in the Western Division of Fiji.

Methods

Focus group discussion

The initial focus group discussion was conducted in 2013 alongside a population-based trachoma prevalence survey[14]. We worked in one iTaukei Fijian village in which adults were known to epilate, and following consultation with the village chief, identified adults who had epilated at least every 3 months for at least 1 year, using a snowball sampling approach[15]. Informed written consent was obtained from each participant. A focus group discussion was

convened and open questions were used to draw out each individual's perceptions of factors associated with epilating behaviour. Each participant's eyes were examined for clinical signs of trachoma by a Global Trachoma Mapping Project-certified grader[16] according to the WHO simplified trachoma grading scheme[10], with photographs of the upper eyelid in primary position, and of the conjunctiva taken to allow later independent review.

The focus group moderator used a list of questions (S1 Appendix) to guide discussion about existing knowledge of trichiasis and epilation, and the causes and natural history of common local eye complaints. A Fijian eyecare nurse assisted with the discussion to clarify cultural nuances, and to provide translation if needed. Participants were encouraged to express themselves in the language in which they were most comfortable. The discussion was audiotaped and transcribed as soon as possible after the event, with input from a Fijian translator where necessary.

Questionnaire development

The transcript was analysed independently by two researchers (CM & RB) using conventional content analysis, with the coding unit as an idea or phrase within a sentence, and each unit classified into constituent themes. From the themes derived, we developed a questionnaire for the population-level survey. Variables assessed included basic demographics, kava drinking habits, ophthalmic history, details of eyelash epilation, and symptoms or factors which influenced the decision to epilate (S2 Appendix). The questionnaire was designed for use with the Open-Data-Kit Collect (https://opendatakit.org) survey data collection platform for Android smartphones.[17,18]

Prevalence survey study design

The epilation prevalence survey was conducted in 2015. A cross-sectional cluster random sampling methodology was employed to select individuals aged ≥15 years in the Western Division of Fiji, with villages or settlements used as the primary sampling unit (cluster) and the household used as the secondary sampling unit. Clusters were selected randomly from a list of all non-urban communities in the Western Division using a probability proportional to size methodology. Rural communities in Fiji commonly have one majority ethnicity, and the organisational structures between ethnic groups are distinct and easily identifiable.

Assuming a design effect of 2, we estimated 768 adults over the age of 15 years would be needed to have 95% confidence of detecting a community prevalence of eyelash epilation in adults of 10% with a precision of +/- 3%. Based on the number of available adults per household in previous surveys and assuming 30 households would be surveyed per day, we estimated the required sample size would be achieved from 26 clusters.

Sampling and examination

In selected clusters, households were randomly selected from a list created on the day of survey in collaboration with local headmen, village chiefs, or healthcare workers. Following consent from the head of household, all those aged ≥15 years resident in selected households were invited to participate. Consenting participants were interviewed individually in their households. Questionnaire responses were recorded electronically in the smartphone application. Those who reported epilating their eyelashes at least once within the past year were invited for ocular examination and photographs of the upper eyelid in primary position, and of the conjunctiva. Consenting participants' eyes were examined and graded using the WHO simplified trachoma grading system by field workers trained in trachoma grading.[10] Photographs were

collected according to a standardised protocol[19] using a Nikon D60 SLR camera with specialised macro lens to allow retrospective grading of the clinical findings.

Ethical clearance and consent

Ethical approval was obtained from the research ethics committees at the London School of Hygiene & Tropical Medicine (reference numbers 012–354 & 9621) and the Fijian Ministry of Health and Medical Services. Local health workers were contacted in advance of the survey to allow community sensitisation. Survey teams engaged in sevu-sevu (a traditional gift of kava roots) with village leaders where appropriate. Written informed consent was obtained from all participants, with a thumbprint considered acceptable in those unable to provide a signature. Information sheets and consent forms were provided in English and Fijian language, and the local nurse provided translation where needed. Participants were advised that they could withdraw from the survey at any time without adverse consequence to them. A parent or guardian provided informed consent and was always present for those aged 15–17 years as well as individuals with mental or physical disabilities. Any individuals found to have ocular pathologies were referred to the nearest eyecare centre using a standard referral form. All data were anonymised and stored on a secure cloud-based server.

Data analysis

Photographs were independently graded by two experienced trachoma graders masked to the clinical assessments. Prevalence estimates were adjusted for age and sex using the 2007 census of Fiji.[11] Confidence intervals were calculated by bootstrapping adjusted cluster-level estimates. A two-level random-effects logistic regression analysis was performed to create a causal risk factor model, against the binary outcome of the presence or absence of the behaviour of eyelash epilation at individual level, accounting for clustering at household- and cluster-level. Variables were assessed for collinearity by tabulation and evaluation with a Mantel-Haenszel Chi^2 test. Variables statistically significant at the $p = 0.10$ level (Wald's test) on univariable analysis were considered for the multivariable model. Variables in the multivariable model were assessed by stepwise inclusion, with factors retained in the model if they reached significance at the $p<0.05$ level (Likelihood ratio test). Data were analysed using Stata version 13.1 (StataCorp, College Station, TX, USA).

Results

Focus group discussion

The focus group was carried out in October 2013. Focus group participants were 6 iTaukei Fijians inhabiting one village in the southern part of the Western Division of Fiji. The group was composed of 2 male construction labourers and 4 female housewives with a median age of 42 years [range 20–53 years]. The village where the focus group occurred was considered by residents to be of wholly iTaukei Fijian ethnicity. No participants were found to have any sign of trachoma on clinical examination.

Transcript analysis

The points discussed during the session fell into 4 themes: motivation for epilation, perceived predispositions to eye symptoms, the Fijian eyecare culture, and a description of the epilation process. The themes and categories derived from the data are shown in Fig 1.

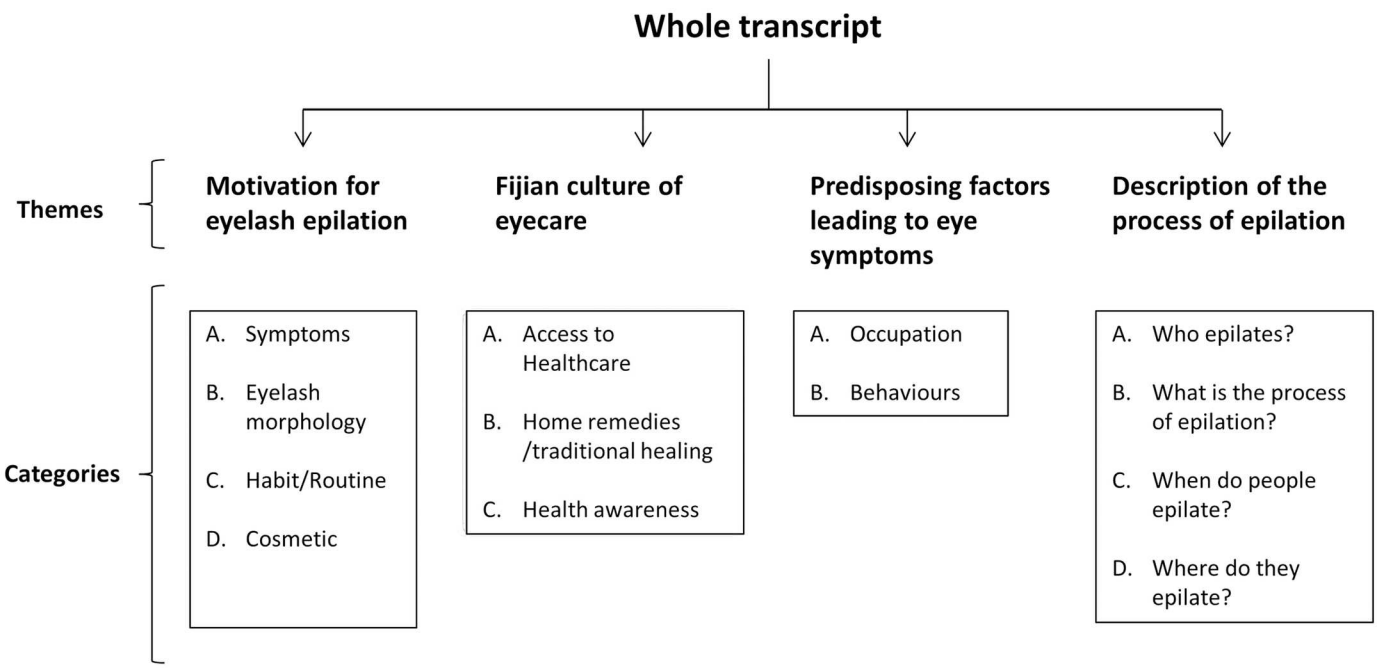


Fig 1. Categories and themes derived from the transcript of the focus group of epilating individuals, Western Division, Fiji, 2013.

doi:10.1371/journal.pntd.0005277.g001

Several themes were identified as perceived motivations for eyelash epilation. In each case, transcript extracts have been provided to support the extracted theme. The most commonly cited symptom that could be relieved by epilation was itchiness.

- "When it's itchy, I'll start pulling the hair out".

The group felt that eyelashes targeted for epilation were abnormal, being short and sharp and able to be removed painlessly.

- "the eyelashes that we pull out, they are different from the normal one."
- "they are prickly. It's only seen from pigs, swines, they have straight, short, sharp eyelashes".

The group described a traditional method of "threading" using fibres from coconut husks, the technique for which was demonstrated during the discussion.

- "They use a coconut husk like that (demonstrates)"
- "And they don't use the pluckers (tweezers)"

Occasionally people would take out large numbers of eyelashes at one time:

- "I pulled out about 120 from my eyes, both of them, with the small ones. Only that was the first time I did that"

Some participants felt that epilation was a habit, but others felt it was more a routine part of their culture.

- "We check our eyes after two, three months. . ., then when you start with it, then it does become a habit"
- "it's a habit, once you start you can't stop so you try to get it out every time"

The participants described good access to and engagement with healthcare services through pharmacists or hospitals when deemed appropriate.

- "we're educated now, we're trying to go to the hospitals. Before they did not go to the hospitals."

Participants described eyecare home remedies and long-held practices

- "actually that was our old people that has been sent from this generation to that generation downwards. It has been, from that time till now we do that for pain relief"
- "In some villages we Fijians, we use herbs. If someone has an eye problem or so he gets these and then we just drop them into the eye and then off they go".

Participants believed epilation-inducing symptoms could be precipitated by occupational exposures, such as sunlight and dust.

- "(it can be brought on by) not using safety goggles and working out in the hot sun, and then out on the roadside".

Some felt that symptoms were associated with the iTaukei cultural practice of drinking kava, as well as the long nights associated with its consumption

- "one thing is kava, drinking kava, you have more time drinking of kava and less time sleeping."

Prevalence survey

The population-based survey was carried out from July-September 2015. 695 participants aged ≥15 years from 457 households in 23 clusters consented to participate. Three clusters could not be reached due to logistical issues at the time of survey. 16 clusters were considered to be of iTaukei ethnicity, 3 clusters were considered Indo-Fijian ethnicity, and 4 clusters did not have a clear majority ethnic group. 512 of 695 (74%) participants were female. 437 (63%) participants were ethnic iTaukei and 235 (34%) participants were ethnic Indo-Fijian. The median age of study participants was 43 years (IQR 30–56; total range 15–88).

125 (18%) of the 695 individuals interviewed reported epilating their eyelashes at least once within the past year. The overall sex- and age-adjusted prevalence estimate of epilation behaviour in those aged ≥15 years in the Western Division was 8.6% (95% CI 5.7–11.3%).

Of the 125 individuals reporting epilation, 124 (99%) consented to examination and conjunctival photography. In 4 of 124 (3%) consenting participants, one or both eyelids were unable to be everted due to discomfort. On examination, no cases of trichiasis were identified, but one suspected case of TS was identified. On subsequent analysis of conjunctival photographs by two independent trachoma graders, no cases of trichiasis or definite TS were identified, with the one clinically suspected TS case thought to have (at best) equivocal evidence of conjunctival scar (Fig 2). Therefore, either none or only one of those who epilated had any evidence of cicatricial trachoma (TS or TT).

Description of epilation habits

Data on the 125 epilators are shown in Table 1. The most commonly reported reason for epilating was eye itchiness (111/ 125 epilators, 89%). 80 (64%) reported that they removed >10 eyelashes each time they epilated, and 80 (64%) reported that they epilated on average every 1–3 months. Front of eye and everted lid photographs from epilators with varying frequencies of epilation behaviour are shown in Fig 3.

Fig 2. Conjunctival photograph of the individual felt to demonstrate equivocal evidence of conjunctival scar, Western Division, Fiji, 2015.

doi:10.1371/journal.pntd.0005277.g002

Risk factor analysis

Table 2 shows the univariable analysis of each potential risk factor against the outcome of eyelash epilation behaviour. Responses relating to a history of watery eye or eye discharge in the previous week were omitted from analyses because of translation difficulties during the survey.

Multivariable model

Results of the two-level multivariable model are shown in Table 3. In the full model, being a regular epilator was associated with being iTaukei (rather than any other ethnicity) (OR 6.0 95%CI 2.6–13.9), and female gender (OR 4.1 95%CI 2.0–8.6). In addition, those who epilated had a higher odds of reporting being a kava drinker (OR 1.7 95%CI 1.1–2.7).

When the reported frequency of kava drinking was considered in the full model, a higher frequency was independently associated with increased odds of being a regular epilator, with those reporting drinking kava daily having odds 4.9 times higher than those who reported drinking kava less than monthly or not at all (OR 4.9, 95%CI 1.6–15.2).

Despite being significant on univariable analysis, age was not associated with regular epilation in the final model. Of note, the effect of age was markedly decreased when the frequency of kava drinking was included in the model, suggesting that this was in part explained by an

Table 1. Characteristics of eyelash epilation among 125 epilators, Western Division, Fiji, 2015.

Description of epilation	n(%)[a]
Number of lashes removed	
Most or all	3(2.4)
>10	80(64.0)
2–10	40(32.0)
1	2(1.6)
Frequency of epilation	
At least weekly	4(3.2)
At least monthly	16(12.8)
Every 1–3 months	80(64.0)
Less than every 3 months	25(20.0)
Median age at first epilation (years)	29(IQR 20–36)
Reasons for epilating	
Itchiness	111(88.8)
Feeling of dust	76(60.8)
Habit	54(3.2)
Abnormal eyelash	37(29.6)
Cosmetic reasons	4(3.2)
Tradition	0(0.0)

[a] Total number of self-reported eyelash epilators = 125

doi:10.1371/journal.pntd.0005277.t001

increased frequency of kava drinking in younger participants. Eye itchiness in the preceding week was strongly associated with being an epilator on univariable analysis (OR 4.5 95%CI 2.8–7.1), but was not included in the final model as this was collinear with kava drinking.

The effect of the frequency of kava drinking on epilation was not confounded by ethnicity, and there was no evidence of interaction between ethnicity and kava drinking frequency on epilation (Likelihood Ratio Test, p = 0.44).

Discussion

We have documented the presence of a common behaviour in Fiji that has not previously been described in the literature. Eyelash epilation in the presence of the distorted eyelid morphology associated with cicatricial trichiasis is a frequent finding in trachoma-endemic populations [9,20–22]. By contrast, individuals in both phases of this study carried out regular epilation in an area where little, if any, trichiasis is found. According to the WHO simplified trachoma grading system, evidence of recent removal of in-turned lashes should be graded as TT. However, it is difficult to provide guidance on how a grader should determine whether an already-epilated lash was misdirected while it was still in situ. Additionally, in the simplified grading scheme, the conjunctiva does not need to be examined to assign a grade of TT. According to the current system, then, individuals who epilate in-turned eyelashes would be correctly graded as having TT even if they have trichiasis for reasons unrelated to trachoma. It is possible, therefore, that this local practice of eyelash epilation in the absence of TT (as demonstrated by our 2013 prevalence survey data)[14] had a significant impact on the 2012 estimate of TT prevalence in Western Division. Our adjusted prevalence estimate of regular epilation in Western Division (8.6% of those aged ≥15 years) is very similar to the 2012 TT prevalence estimate (8.7%) in the same population[2]. This may account for the apparent discrepancy between TT prevalence estimates (8.7% in 2012; 0% in 2013) and the lack of people presenting locally for

Fig 3. Front-of-eye and conjunctival photographs of individuals who removed (Bottom) 2–10 eyelashes, (Middle) >10 eyelashes (with pterygium), or (Top) most or all of the eyelashes; Western Division, Fiji, 2015.

doi:10.1371/journal.pntd.0005277.g003

TT surgery. Two individuals with TT reportedly presented to surgical outreach clinics in Fiji between 2011 and 2013, neither of whom received corrective surgery[23].

The origin of the epilation behaviour in this population is unclear, though our data help to generate hypotheses and exclude some potential explanations. The most commonly described symptom motivating epilation was itchiness, whereas trichiasis is more commonly associated with painful, watery or purulent eyes, or blepharospasm[6]. Neither the natural history nor the examination findings in our subjects were consistent with other ocular pathologies that can lead to trichiasis, such as involutional changes linked to senescence, marginal entropion from chronic inflammation due to blepharitis or meibomian gland disease, or distichiasis, when an extra row of maldirected eyelashes is present[24]. These are all conditions that may prompt individuals to epilate, but are considered rare at population level. A psychological cause (such

Table 2. Univariable multi-level random effects logistic regression model for associations of eyelash epilation, Western Division, Fiji, 2015.

Variable		Total (%)	Number of epilators (%)	Odds Ratio	95% Confidence Interval	p-value[a]
Ethnicity	Indo-Fijian	235 (33.8)	7 (5.6)	1	-	-
	iTaukei	437 (62.9)	117 (93.6)	10.7	4.6–25.1	<0.001
	Other[b]	23 (3.3)	1 (0.8)	1.8	0.2–15.9	0.615
Gender	Male	183 (26.3)	14 (11.2)	1	-	-
	Female	512 (73.7)	111 (88.8)	2.9	1.6–5.3	0.001
Age (years)	15–24	93 (13.4)	22 (17.6)	3.4	1.7–7.1	0.042
	25–34	154 (22.2)	33 (26.4)	2.5	1.3–4.8	
	35–44	118 (17.0)	23 (18.4)	2.0	1.0–4.1	
	45–54	136 (19.6)	24 (19.2)	2.0	1.0–4.0	
	55+	194 (28.0)	23 (18.4)	1	-	
Frequency of kava drinking	Daily	38(5.5)	8(6.4)	2.1	0.8–5.2	*0.0608*
	At least weekly	137 (19.7)	33(26.4)	1.8	1.0–3.0	
	More than monthly but less than weekly	82 (11.8)	22 (17.6)	1.8	0.9–3.3	
	Less than monthly	39 (5.6)	6 (4.8)	0.7	0.3–1.8	
	Never	399(57.4)	56(44.8)	1		
Eye itchiness in the previous week	No	406 (58.4)	34 (27.2)	1	-	-
	Yes	289 (41.6)	91 (72.8)	4.5	2.8–7.1	<0.001
Eye redness in the previous week	No	492 (70.8)	72 (57.6)	1	-	-
	Yes	203 (29.2)	53 (42.4)	1.7	1.1–2.7	0.015
Past ophthalmic history	None	611(87.9)	120(96.0)	1		0.6737
	Blind	2(0.3)	0(0.0)	-		
	Current Cataract	12(1.7)	3(2.4)	1.2	0.3–4.9	
	Previous cataract surgery	4(0.6)	0(0.0)	-		
	Treated for active trachoma	2(0.3)	1(0.8)	2.4	0.1–38.7	
	Traumatic ocular injury	4(0.6)	1(0.8)	2.0	0.2–23.5	
Total		**695**	**125**	**-**	**-**	**-**

[a] Wald's test

[b] European, Chinese, Rotuman, Other Pacific islanders

doi:10.1371/journal.pntd.0005277.t002

as the impulse control disorder trichotillomania, whereby the individual cannot control urges to pull out their own hair) was inconsistent with the descriptions given by the focus group.

Drinking of kava, and particularly drinking kava often, was a strong independent risk factor for regular epilation in our population. Kava (*Piper methysticum*) is a perennial plant that can be used to prepare a non-alcoholic drink by mixing the ground root and stem bases with water

Table 3. Multivariable multi-level random effects logistic regression model for associations of eyelash epilation, Western Division, Fiji, 2015.

Variable	Adjusted Odds Ratio	95% Confidence interval	p-value[a]
Female sex	4.1	1.9–8.0	<0.0001
iTaukei ethnicity [b]	6	3.0–18.6	<0.0001
Kava drinking frequency			
Daily	4.5	1.6–14.6	0.0096
At least weekly	1.9	1.1–3.5	
At least monthly	2.2	1.0–3.6	
Less than monthly, or never	1	-	

[a] Likelihood ratio test

[b] Compared to Indo-Fijian, European, Chinese, Rotuman, Other Pacific islanders

doi:10.1371/journal.pntd.0005277.t003

[25]. Kava has been used in South Pacific communities for centuries, for medicinal, social and cultural purposes[26]. The active ingredients are kavalactones, which are associated with eye itchiness as a side effect. Participants in the focus group felt that consumption directly precipitated eye symptoms; others felt symptoms could be brought on by sleep deprivation associated with its use. In a randomised controlled trial in Tonga, kava drinkers were reported to have "red, irritated eyes and increased photosensitivity during periods of heavy drinking"[27].

It seems likely that kava may produce eye symptoms, but the potential mechanism for relief through epilation is unclear. It is possible that it could be a form of distraction, either psychologically, or physically through the pain commonly associated with epilation. Importantly, although drinking kava was strongly associated with epilation, 56 (45%) of the 125 epilators reported never drinking kava. This supports the idea that in Fijian custom a variety of eye symptoms might be considered to be amenable to epilation, with itchiness from kava drinking being just one. It is possible that epilation in this context could also represent a cultural practice reflective of a time when trichiasis was more common and that this persisted even after trichiasis became rare.

As further evidence of a cultural determinant of this behaviour, in the full model, we found that iTaukei individuals had 6.0 times greater odds of being an epilator than those of any other ethnicity. The major ethnic groups in Fiji are iTaukei and Indo-Fijian—both with distinct cultures, practices, beliefs and languages. iTaukei are predominantly Christian and speak an indigenous language, whereas Indo-Fijians are mainly Hindu or Muslim and speak a local variant of Hindi. The overall population of Fiji comprises 56.8% iTaukei and 37.5% Indo-Fijian, with the remainder being a mixture of European, Chinese, Rotuman and other Pacific Islanders.[11]

Although its origins are elusive, significant differences are seen between the epilation behaviour described here, and the anticipated behaviour if epilation was related to trichiasis. Both the number of eyelashes removed and the frequency of epilation found in this population are noticeably different from those normally reported in the context of TT. A study in Ethiopia, for example, found that among individuals with trichiasis who self-managed symptoms by epilating, there was a median of 2 eyelashes touching the eye (interquartile range 1–5),[9] whereas in our population, the majority of epilators (66%) removed more than 10 eyelashes each time. In addition, the same Ethiopian study found that 96% epilated at least once per month, with 51% epilating at least once per week,[9] consistent with the need to relieve symptoms from eyelash regrowth, whereas in Fiji we found only 16% of epilators epilated at least once per month, with only 3% epilating at least once per week.

As is commonly found in household surveys carried out during the day, we have an under-representation of males in the survey. As only those present at the household at the time of survey were enumerated (rather than all household inhabitants whether present or not), it is not possible to determine the true extent to which men were under-represented in the households sampled. It is possible that, given that both survey field researchers were female, there might have been higher uptake from female community members. This could have potentially been addressed with a stronger approach to community sensitisation in advance of the surveys. Our focus group discussion took place in an iTaukei village because when it was conducted we had not heard that epilation was also practised by other ethnic groups; it is possible that this influenced the choice of possible risk factors assessed at population level. A further potential weakness of our work is the inherent difficulty in standardising the diagnosis of TS, which was not a feature of the training and standardisation of graders carried out as part of the Global Trachoma Mapping Project[16]. We tried to compensate for this last weakness by taking conjunctival photographs and having the images reviewed by highly experienced trachoma researchers —though even here, one image (Fig 2) was controversial.

In this study we have highlighted an epilation behaviour that could significantly bias TT prevalence estimates. We conclude that epilation in the absence of trichiasis has the potential to impact programme planning for trachoma elimination in countries where this practice is common. Further studies in Pacific populations where kava is consumed should be undertaken to see whether the relationship with kava drinking seen in our study is true in other settings. Given the difficulties inherent in determining whether epilated eyelashes were inturned, consideration should be given in future surveys to require the presence of TS in epilated eyelids in order to diagnose TT.

Supporting Information

S1 Appendix. Questions used by the focus group moderator to guide discussion about existing knowledge of trichiasis and epilation, and the causes and natural history of common local eye complaints, Western Division, Fiji, 2013.
(DOCX)

S2 Appendix. Data collected in the questionnaire by the survey team, Western Division, Fiji, 2015.
(DOCX)

S1 Checklist. STROBE checklist for reports of observational studies.
(DOCX)

Author Contributions

Conceptualization: CM CY RB UM KN NC MM AWS.

Data curation: CM CY.

Formal analysis: CM CY CH.

Funding acquisition: CM RB MM.

Investigation: CY NC UM KN MRQ CW.

Methodology: CM CY RB CW CH.

Project administration: CM RB MM MRQ.

Resources: MK ER LC MRQ.

Software: CM CY RB MM.

Supervision: AWS DCWM LC MK ER.

Validation: CM RB MM KN MRQ LC ER.

Visualization: CM CY CHR RB MM.

Writing – original draft: CM CY RB MM DCWM CH.

Writing – review & editing: AWS DCWM UM KN CH CW.

References

1. Bourne RRA, Stevens GA, White RA, Smith JL, Flaxman SR, Price H, et al. Causes of vision loss worldwide, 1990–2010: a systematic analysis. Lancet Glob Heal. Elsevier; 2013; 1: e339–49.

2. World Health Organization. Report of the 3rd Global Scientific meeting on trachoma, Johns Hopkins University, Baltimore, MA, 19–20 July 2010. Geneva: World Health Organization; 2010.
3. World Health Organization. Validation of elimination of trachoma as a public health problem (WHO/HTM/NTD/2016.8). Geneva: World Health Organization; 2016.
4. Francis V, Turner V. Achieving community support for trachoma control (WHO/PBL/93.36). Geneva: Geneva: World Health Organization; 1993.
5. Merbs S, Resnikoff S, Kello S, et al. Trichiasis surgery for trachoma (2nd Ed). Geneva: World Health Organization; 2015.
6. Palmer SL, Winskell K, Patterson AE, Boubacar K, Ibrahim F, Namata I, et al. "A living death": a qualitative assessment of quality of life among women with trichiasis in rural Niger. Int Health. 2014; 6: 291–7. doi: 10.1093/inthealth/ihu054 PMID: 25125577
7. Habtamu E, Wondie T, Aweke S, Tadesse Z, Zerihun M, Zewudie Z, et al. The Impact of Trachomatous Trichiasis on Quality of Life: A Case Control Study. PLoS Negl Trop Dis. United States; 2015; 9: e0004254.
8. Rajak SN, Habtamu E, Weiss HA, Kello AB, Gebre T, Genet A, et al. Surgery Versus Epilation for the Treatment of Minor Trichiasis in Ethiopia: A Randomised Controlled Noninferiority Trial. PLoS Med. 2011; 8: e1001136. doi: 10.1371/journal.pmed.1001136 PMID: 22180731
9. Rajak SN, Habtamu E, Weiss HA, Bedri A, Gebre T, Genet A, et al. Epilation for Trachomatous Trichiasis and the Risk of Corneal Opacification. Ophthalmology. 2012; 119: 84–9. doi: 10.1016/j.ophtha.2011.06.045 PMID: 21975041
10. Thylefors B, Dawson CR, Jones BR, West SK, Taylor HR. A simple system for the assessment of trachoma and its complications. Bull World Health Organ. 1987; 65: 477–83. PMID: 3500800
11. Fiji Bureau of Statistics. 2007 Census of Population. In: Suva, Fiji [Internet]. 2007 [cited 24 Jun 2015]. http://www.statsfiji.gov.fj/index.php/2007-census-of-population
12. Mathew AA, Keeffe JE, Le Mesurier RT, Taylor HR. Trachoma in the Pacific Islands: evidence from Trachoma Rapid Assessment. Br J Ophthalmol. 2009; 93: 866–70. doi: 10.1136/bjo.2008.151720 PMID: 19174394
13. Kama M, Cama A, Rawalai K, Koroivueta J. Active Ocular Trachoma In Fiji- A Population Based Prevalence Survey. Fiji J Public Heal. 2013; 2: 11–17.
14. Macleod CK, Butcher R, Mudaliar U, Natutusau K, Pavluck AL, Willis R, et al. Low Prevalence of Ocular Chlamydia trachomatis Infection and Active Trachoma in the Western Division of Fiji. Senok A, editor. PLoS Negl Trop Dis. Public Library of Science; 2016; 10: e0004798.
15. Biernacki P, Waldorf D. Snowball Sampling. Problems and Techniques of Chain Referral Sampling. Sociological Methods & Research. SAGE Publications Ltd; 1981. pp. 141–163.
16. Solomon AW, Pavluck A, Courtright P, Aboe A, Adamu L, Alemayehu W, et al. The Global Trachoma Mapping Project: methodology of a 34-country population-based study. Ophthalmic Epidemiol. 2015; 22: 214–225. doi: 10.3109/09286586.2015.1037401 PMID: 26158580
17. Hartung C, Anokwa Y, Brunette W, Lerer A, Tseng C, Borriello G. Open Data Kit: Tools to Build Information Services for Developing Regions. Proc Int Conf Inf Commun Technol Dev. 2010; 1–11.
18. Pavluck A, Chu B, Mann Flueckiger R, Ottesen E. Electronic data capture tools for global health programs: evolution of LINKS, an Android-, web-based system. PLoS Negl Trop Dis. Public Library of Science; 2014; 8: e2654.
19. Stare D, Harding-Esch E, Munoz B, Bailey R, Mabey D, Holland M, et al. Design and baseline data of a randomized trial to evaluate coverage and frequency of mass treatment with azithromycin: the Partnership for Rapid Elimination of Trachoma (PRET) in Tanzania and The Gambia. Ophthalmic Epidemiol. England; 2011; 18: 20–29.
20. Melese M, West ES, Alemayehu W, Munoz B, Worku A, Gaydos CA, et al. Characteristics of trichiasis patients presenting for surgery in rural Ethiopia. Br J Ophthalmol. 2005; 89: 1084–1088. doi: 10.1136/bjo.2005.066076 PMID: 16113353
21. West ES, Munoz B, Imeru A, Alemayehu W, Melese M, West SK. The association between epilation and corneal opacity among eyes with trachomatous trichiasis. Br J Ophthalmol. 2006; 90: 171–174. doi: 10.1136/bjo.2005.075390 PMID: 16424528
22. West S, Alemayehu W, Munoz B, Gower EW. Azithromycin prevents recurrence of severe trichiasis following trichiasis surgery: STAR trial. Ophthalmic Epidemiol. England; 2007; 14: 273–277.
23. Lees J, McCool J, Woodward A. Eye health outreach services in the Pacific Islands region: an updated profile. N Z Med J. 2015; 128: 25–33. Available: http://www.ncbi.nlm.nih.gov/pubmed/26367510
24. Choo P. Distichiasis, trichiasis, and entropion: advances in management. Int Ophthlmol Clin. 2002; 42: 75–87.

25. McDonald D, Jowitt A. Kava in the Pacific Islands: a contemporary drug of abuse? Drug Alcohol Rev. 2000; 19: 217–227.

26. Davis R, JF B. Kava (Piper methysticum) in the South Pacific: its importance, methods of cultivation, cultivars, diseases and pests. Canberra: Australian Centre for International Agricultural Research Report Series; 1999.

27. Ruze P. Kava-induced dermopathy: a niacin deficiency? Lancet. 1990; 335: 1442–5. PMID: 1972218

Forms were prepared in .xls format and converted to .xml ODK readable format at http://opendatakit.org/xiframe.

Part	Question	Answer	Format
1	Start time		
2	Enter the initials of the person entering this data	Initials	Free text
3	Are you satisfied the person is informed of the purpose of the study and takes part willingly?	1 Yes 0 No	Select one
4	Please select the island group you are working on.	1 Santa Cruz 2 Utupua 3 Vanikoro 4 Bellona 5 Other	Select one
5	If other, please enter name	Island name	Free text
6	Please select the cluster you are surveying.	1 Malo 2 Nonia 3 Matu 4 No'oka 5 Gaito 6 Nembao 7 Matembo 8 Tanibili 9 Lale 10 Lavaka 11 Buma 12 Emua 13 Bellona 14 Other	Select one
7	If other, please enter name	Cluster name	Free text
8	Please enter the household number	Household number	Numeric
9	Please enter the participants initials	Participant initials	Free text
10	Please enter the participants age in full years	Age	Numeric
11	Please enter the sex of the participant	1 Male 2 Female	Select one
12	Please enter the participant ID	Numeric	SB000-000
13	Please re-enter the participant ID	Numeric	SB000-000, must match [12]
14	Is there evidence of TT in the right eye?	0 No 1 Yes 2 Unable to examine	Select one
15	Is there evidence of TF in the right eye?	0 No 1 Yes 2 Unable to examine	Select one
16	Is there evidence of TI in the right eye?	0 No 1 Yes 2 Unable to examine	Select one
17	Was a swab collected?	0 No 1 Yes	Select one
18	Enter the first frame ID	Numeric	DSC_0000
19	Enter the second frame ID	Numeric	DSC_0000

20	Enter the third frame ID	Numeric	DSC_0000
21	Is there evidence of TT in the left eye?	0 No 1 Yes 2 Unable to examine	Select one
22	Is there evidence of TF in the left eye?	0 No 1 Yes 2 Unable to examine	Select one
23	Is there evidence of TI in the left eye?	0 No 1 Yes 2 Unable to examine	Select one
24	Was a dried blood spot collected?	0 No 1 Yes	Select one
25	Add any relevant notes	Note	Free text

London School of Hygiene & Tropical Medicine
Keppel Street, London WC1E 7HT
United Kingdom
Switchboard: +44 (0)20 7636 8636

www.lshtm.ac.uk

Observational / Interventions Research Ethics Committee

Anthony Solomon
Senior Lecturer
CR / ITD
LSHTM

9 August 2013

Dear Dr. Solomon,

Study Title: **Solving the clinical enigma of trachoma in the Pacific Islands**
LSHTM ethics ref: **6360**

Thank you for your letter of 8 August 2013, responding to the Observational Committee's request for further information on the above research and submitting revised documentation.

The further information has been considered on behalf of the Committee by the Chair.

Confirmation of ethical opinion

On behalf of the Committee, I am pleased to confirm a favourable ethical opinion for the above research on the basis described in the application form, protocol and supporting documentation as revised, subject to the conditions specified below.

Conditions of the favourable opinion

Approval is dependent on local ethical approval having been received, where relevant.

Approved documents

The final list of documents reviewed and approved by the Committee is as follows:

Document	Version	Date
LSHTM ethics application	2	08/08/2013
Proposal	7	24/04/2013
Information Sheet		31/01/2013
Consent form		31/01/2013

After ethical review

Any subsequent changes to the application must be submitted to the Committee via an E2 amendment form. All studies are also required to notify the ethics committee of any serious adverse events which occur during the project via form E4. At the end of the study, please notify the committee via form E5.

Yours sincerely,

Professor John DH Porter
Chair
ethics@lshtm.ac.uk
http://intra.lshtm.ac.uk/management/committees/ethics/

London School of Hygiene & Tropical Medicine
Keppel Street, London WC1E 7HT
United Kingdom
Switchboard: +44 (0)20 7636 8636

www.lshtm.ac.uk

Observational / Interventions Research Ethics Committee

LSHTM

13 May 2015

Dear

Study Title: Solving the Clinical Enigma of Trachoma in the Pacific Islands : Phase Two

LSHTM Ethics Ref: 8402

Thank you for responding to the Observational Committee's request for further information on the above research and submitting revised documentation.

The further information has been considered on behalf of the Committee by the Chair.

Confirmation of ethical opinion

On behalf of the Committee, I am pleased to confirm a favourable ethical opinion for the above research on the basis described in the application form, protocol and supporting documentation as revised, subject to the conditions specified below.

Conditions of the favourable opinion

Approval is dependent on local ethical approval having been received, where relevant.

Approved documents

The final list of documents reviewed and approved by the Committee is as follows:

Document Type	File Name	Date	Version
Covering Letter	150508_LSHTM_ethics_response		
Information Sheet	150508_SLB_seroepidemiology_consent_form_v2	08/05/2015	v4
Information Sheet	150508_SLB_seroepidemiology_information_sheet_v2	08/05/2015	v4

After ethical review

The Chief Investigator (CI) or delegate is responsible for informing the ethics committee of any subsequent changes to the application. These must be submitted to the Committee for review using an Amendment form. Amendments must not be initiated before receipt of written favourable opinion from the committee.

The CI or delegate is also required to notify the ethics committee of any protocol violations and/or Suspected Unexpected Serious Adverse Reactions (SUSARs) which occur during the project by submitting a Serious Adverse Event form.

At the end of the study, the CI or delegate must notify the committee using an End of Study form.

All aforementioned forms are available on the ethics online applications website and can only be submitted to the committee via the website at: http://leo.lshtm.ac.uk

Additional information is available at: www.lshtm.ac.uk/ethics

Yours sincerely,

Professor John DH Porter
Chair

ethics@lshtm.ac.uk
http://www.lshtm.ac.uk/ethics/

No: HRC13/18

National Health Research & Ethics Committee
Solomon Islands Ministry of Health & Medical Services

Research Certificate

To Dr. Anthony Solomon
London School of Hygiene and Tropical Medicine

The National Health Research & Ethics Committee (NHREC) of the Ministry of Health & Medical Services, Solomon Islands has in August 2013, approved your application to do research titled ***"Solving the clinical enigma of trachoma in the Pacific Islands".***

You are hereby granted permission to conduct your research in Solomon Islands as for 2013. This approval is for the one-time conduction of your research and any repetition and/or extension of this research will need further NHREC approval.

Dalipanda

Dr Tenneth Dalipanda
Chairman, NHREC

23/08/2013

Date

No: HRC15/03

National Health Research & Ethics Committee
Solomon Islands Ministry of Health & Medical Services

Research Certificate

To Dr. Chrissy h. Roberts,
London School of Hygiene & Tropical Medicine,
United Kingdom.

The National Health Research & Ethics Committee (NHREC) of the Ministry of Health & Medical Services, Solomon Islands has deliberated on 4th March 2015 and has approved your application to your research titled ***"Seroepidemiology of Chlamydia trachomatis in the outer Solomon Islands".***

You are hereby granted permission to conduct your research in Solomon Islands for the year 2015. This approval is for the one-time conduction of your research and any amendments, repetition and/or extension of this research will need further NHREC approval. A progressive report is to be submitted to the committee for the duration of this approved term of research. Failure to abide to the above terms will result in suspension or discontinuation of approval.

NATIONAL HEALTH TRAINING & RESEARCH INSTITUTE
INITIAL:
DATE:
MINISTRY OF HEALTH & MEDICAL SERVICES

Dr Christopher Becha
Chairman, NHREC

5/03/2015
Date

GOUVERNEMENT
DE LA
REPUBLIQUE DE VANUATU
MINISTERE DE LA SANTE

GOVERNMENT
OF THE
REPUBLIC OF VANUATU
MINISTRY OF HEALTH

Director General

Ref: MOH/DG 01/21- GKT/lr

8 April 2016

Mrs. Fasihah Taleo
Neglected Tropical Disease Coordinator
WHO

Dear Mrs. Taleo

Subject: Ethical Clearance for Research Proposal: Extended mapping of ocular Chlamydia trachomatis infection in the Pacific Islands

Thank you for your research proposal submission on the above topic.

The Ministry of Health endorses your research proposal in Vanuatu and looks forward to the submission of the findings of this survey.

Yours sincerely,

REPUBLIC OF VANUATU
DIRECTOR GENERAL OF HEALTH
DIRECTEUR GENERAL DE LA SANTÉ
REPUBLIQUE DE VANUATU

George Taleo
Director General

cc: 1st PA to the Hon Minister of Health
OIC Public Health

PRIVATE MAIL BAG 042, PORT VILA, VANUATU – PHONE: (678) 22545 – FAX: (678) 26113 – TELEX: 1040 VANGOV NH

Seroepidemiology of *Chlamydia trachomatis* in the Solomon Islands.

Local Principal Investigator: Oliver Sokana, Solomon Island Ministry of Health

Consent form (v.14.01.15)

Participant name:……

Participant age:……..

[Affix ID label here]

The information sheet concerning this study has been read to **me / my dependent [CONSENTER TO DELETE AS NECESSARY]**, and I understand what will be expected of someone taking part in this study.

I understand that an individual taking part in this study may withdraw from it at any time without giving a reason and that this will not affect his/her normal care. My questions concerning this study have been answered.

- ☐ I give consent for the above to take part in this study.
- ☐ I give consent for blood and eye samples from the above to be used for the purposes of this study
- ☐ I give consent for photographs of the above to be used for the purposes of this study, which may involve dissemination to relevant external parties and potential publication online.

Signature/thumbprint:

Date:……

[Sign/print here]

If participant is under the age of 18, a parent or guardian should sign this form:

Name:…… Relation:……

##

Witness: I have read this form and the information form to the above person and am sure that he/she has understood what is required of someone enrolling in this study.

Signed:…… Date:……

Solving the clinical Enigma of trachoma in the Pacific Islands: Phase 2

You are being invited to take part in a research study. Before you decide it is important for you to understand why the research is being done and what it will involve. Please take time to read the following information carefully and to talk to others about the study, if you wish. Ask us if there is anything that is not clear or if you would like more information. Take time to decide whether or not you wish to take part.

1. What is the purpose of the study?

After a survey in 2013, we have found large amounts of a disease that looks like trachoma, an eye infection that could cause blindness, but very little infection with a germ called *Chlamydia trachomatis,* which causes this disease in other parts of the world.

In this survey, we would like to use a different method of detecting this germ, to make sure the information from our last survey is correct. We would also like to take photographs so experts can examine whether there are any visible signs of disease that may lead to impaired vision. We may also search for other germs that may be causing this disease in an attempt to work out whether they are harmful or not.

It is important to determine what is causing the disease that looks like trachoma to help inform the Solomon Island Ministry of Health on the most appropriate way to treat this disease.

2. Why have I been chosen?

We are looking for people of any age above 1 year who live in specific villages in Temotu, Rennel and Bellona where our previous survey took place. These villages were selected because high levels of trachoma-like disease were seen here during the past survey. In these villages, we will select households at random and everybody in those households will be offered the opportunity to take part.

3. Do I have to take part?

It is entirely up to you to decide to join the study. We will describe the study and go through this information sheet. If you agree to take part, we will then ask you to sign a consent form. You are free to withdraw at any time, without giving a reason. This will not affect the standard of care you receive.

4. What will happen to me if I take part?

If you decide to take part, we will ask you to come to the centre of the village along with the other members of your household, where our team will greet you and answer any questions you may have before the start of the procedure. We will then:

1. Examine your eyes in the normal position for signs of trachoma-like disease, and take photographs for our records.

2. Turn your eyelid over and examine the underside for signs of trachoma-like disease, and take photographs for our records.

3. Take a swab from the underside of your eyelid.

4. Make a pin-prick in the top of your finger, which will bleed a little. The nurse will then collect a few drops of blood on filter paper

The photographs, blood spots and swabs will be transported to London, UK, where they will by experienced doctors and scientists for evidence of infection with the germ *Chlamydia trachomatis* and evidence of disease.

5. What do I have to do?

If you decide to take part, the examinations listed above (finger prick, eyelid turning, photograph, swab) will be the only ones needed as part of this survey.

6. What are the possible disadvantages and risks of taking part?

There are very few risks to taking part in this survey. The finger pricks and swabs can be uncomfortable. We have tried these procedures many times in the Solomon Islands, and many other parts of the world such as Fiji, Tanzania and The Gambia, and no serious risks have emerged.

7. What are the possible benefits of taking part?

The study will not help you immediately, but the information we get might help improve the treatment of people with trachoma in the Solomon Islands and around the world.

8. What happens when the research study stops?

After the research study stops, the findings of the survey will be published in an easily accessible format. We may store the blood and eye samples for up to 7 years in case anyone should question our results.

Photographs may be used for teaching and research purposes, and may be published in open access format meaning they may become available on the internet. However, if photographs are published, we would ensure that the photographs would not be easily linkable to you as an individual.

9. Will my taking part in the study be kept confidential?

Yes. All information collected about you during the course of the research will be kept strictly confidential.

If you join the study, some parts of your medical records and the data collected for the study may be looked at by authorised persons from the London School of Hygiene & Tropical Medicine. They may also be looked at by representatives of regulatory authorities and by authorised people from the Solomon Islands Ministry of Health to check that the study is being carried out correctly. All will have a duty of confidentiality to you as a research participant and nothing that could reveal your identity will be disclosed outside the research site.

10. What will happen if I don't want to carry on with the study?

You are free to withdraw from the study at any time. If you withdraw from the study, we will destroy all identifiable samples, but we will need to use the data collected up to your withdrawal.

11. What will happen to the results of the research study?

The results of the study will be published, and shared with healthcare bodies in the Solomon Islands, the Western Pacific Region and the wider international community for the purposes of making treatment decisions with the Ministry of Health.

12. Who is organising and funding the research?

The Fred Hollows Foundation are paying for the study. The study is being designed and conducted as collaboration between the Solomon Islands Ministry of Health and the London School of Hygiene & Tropical Medicine.

13. Who has reviewed the study?

This study was given a favourable ethical opinion by the London School of Hygiene & Tropical Medicine Research Ethics Committee and the Solomon Islands Health and Research Ethics Committee.

14. Contact Details

Should you have any questions or worries about the project, please feel free to contact any one of the study investigators:

Oliver Sokana, Solomon Island Ministry of Health
+677-769-1615
osokana@moh.gov.sb

Chrissy h. Roberts, London School of Hygiene & Tropical Medicine
0044-207-927-2913
chrissy.roberts@lshtm.ac.uk

Kelvin Jack, Kukum Clinic
+677-750-0313

Eric Kalae, Lata Hospital
+677-745-5055

A copy of this information sheet will be offered to the leaders of each village and household.

Thank you for taking the time to read this sheet.

Solomon Island Seroepidemiology Survey:
Sample Collection Standard Operating Procedure

Overview

This survey aims to collect infection and clinical data from communities in the Solomon Islands previously found to be hyper-endemic for trachoma, but also to have a low prevalence of current infection with *Chlamydia trachomatis*. The survey aims to describe the prevalence of serological evidence of infection, of infection, and of clinical signs of disease following one round of mass drug administration in communities studied.

All consenting residents over the age of 1 year in randomly selected households will be asked to provide the following:

- Demographic information, including:
  - Location data (village and household location)
  - Name
  - Sex
  - Age (in years)
- Simplified WHO trachoma grade
- Photograph of the eyelid while the eye is in the primary gaze (if graded as TT)
- Photograph of the upper corneal limbus
- Photograph of the everted conjunctiva
- Conjunctival swab x 2
- Dried blood spot

Adults over the age of 18 years will be asked for written consent before taking part. Written consent from a parent or guardian will be required for those under 18 years to take part.

The following staff are expected to be required during the survey:

Staff		
Ophthalmic Nurse (GTMP certified)	1 (OS)	Honiara
Nurse	1 (TBC)	Lata
Village guide	1 (LS)	Lata
Sample manager	1 (RB)	London
Boat driver	1 (TBC)	Lata

Equipment list

Item	Number	Source
Training		
Facilities	1 (suggest provincial guest house)	Lata
Computer	1	London
Swabs	100	London
Filter papers	100	London
Alcohol wipes	100	London
Gloves	100	Honiara
SLR camera	1	London
Sharps bin	1	London
Survey		
Boat	1	Lata
40hp OBM	1	Lata
Fuel	500L	Lata
Tarpaulin	2	Lata
Crate	1	London
Portable freezer	1	Honiara
Dry bag	1	London
Rain coats	5	London
Plastic box for equipment	1	Honiara
Laptop	1	London
Paper replacement forms	500	London
Consent forms	1500 (375 pages @ 4 per page)	London
Information sheets	50 (150 pages @ 3 pages per sheet)	London
Labels (swab, filter paper, form)	4500 (3 per participant)	London
Swabs	4000 (2 per participant)	London
Filter papers	2000 (1 per participant plus excess)	London
Lancets	2000 (1 per participant plus excess)	London
Alcohol wipes	2000 (1 per participant plus excess)	London
Sweets	2000 (1 per participant)	London
Desiccant sachets	3000 (1 per DBS plus shipping container)	London
Small Ziplock bags	2000 (1 per DBS plus excess)	London
Large Ziplock bags	20 (1 per cluster plus excess)	London
Drying mechanism	1	Honiara
Tubes	2000	London
Cryoboxes	20	London
Eskie	1	London
SLR Camera	1	London
Pens	10	Honiara
Ink pad	2	Honiara
Receipt book	1	Honiara
Spare camera battery	3	London
Spare laptop battery	3	London

Phase I: 3 weeks before survey

Step		Task
1	1.1	Arrange procurement of materials
	1.2	Arrange local consignee and clearance
	1.3	Arrange staff and equipment availability
	1.4	Ship equipment and forms
2	2.1	Book flights London to Lata for RB
	2.2	Book flights Honiara to Lata for OS

Phase II:

Step		Task
1	1.1	Meet laboratory staff to arrange freezer storage in Honiara
	1.2	Meet with Oliver to discuss logistics
	1.3	Meet Willie at NRH to arrange shipping and logistics
	1.4	Arrange procurement of tetracycline, gloves, chlorohexamide, wool, alcohol gel for blood spots
	1.5	Arrange onward transit of materials, including freezer, to Santa Cruz
	1.6	Arrange flights to Santa Cruz

Phase III:

Step		Task
1	1.1	Arrange safe transit and storage of equipment
	1.2	Arrange storage space in laboratory
	1.3	Meet with staff in Lata at Rotary Guest House
	1.4	Arrange prior notice for all targeted communities
	1.5	Arrange transport for as much of trip as possible – purchase fuel
2	2.1	Training day am – discuss general purpose of study, discuss study sites, sample size, study requirements
	2.2	Training day pm – discuss proposed methodology, get feedback about feasibility
	2.3	Charge phones, charge camera batteries
	2.4	Begin punching out blood spot cards
3	3.1	Go to nearest cluster (?Matu) and perform first survey site under training conditions
	3.2	Return samples to freezer
	3.3	Discuss points for improvement
	3.4	Keep punching out blood cards
4	4.1	Proceed to first cluster

Phase IV:

Step		Task
1	1.1	Arrange meeting time and place for first survey cluster
	1.2	Travel to first cluster.
	1.3	Meet with village leaders and explain purpose of survey. Gain consent for village enrollment
	1.4	Arrange central site for temporary clinic
	1.5	Select clusters at random from full household list
2	2.1	Village guide to approach first household, explain study and gain consent
	2.2	Consenting participants to approach central site, bringing signed forms with them

3	3.1	Demographic data collected from participants.
	3.2	Place ID labels on form (.1), swab tube (.2 and .3) and blood card (.4)
	3.3	Record ID
4	4.1	Grader to apply alcohol gel and then gloves
	4.2	Take photograph of right upper corneal limbus
	4.3	Examine for TT
	4.4	Sample manager to prepare 2 x swab packets
	4.5	Evert right eyelid
	4.6	Take photograph
	4.7	Grade TF, TI and TS
	4.8	Grader collects first swab
	4.9	First swab passed to sample manager to place in tube (.2)
	4.10	Grader collects second swab
	4.11	Second swab passed to sample manager to place in tube (.3)
	4.12	Sample manager records right eye phenotype
5	5.1	Sample manager records DSC photo ID
6	6.1	Grader checks left eye for TT
	6.2	Grader everts left eyelid
	6.3	Grader examines left eyelid for TF, Ti and TS
	6.4	Sample manager records phenotype
	6.5	Grader removes and discards gloves
7	7.1	Sample manager passes blood card to blood spot station
	7.2	Nurse prepares by washing hands with alcohol gel
	7.3	Nurse puts on gloves
	7.4	Nurse wipes finger of participant
	7.5	Nurse pricks finger
	7.6	Nurse fills 6 extensions on blood card
	7.7	Nurse places blood card on drying stand
	7.8	Nurse provides participant with sweet
	7.9	Nurse confirms blood card collected

Phase V:

Step		**Task**
1	1.1	Swabs immediately transferred to tubes, and stored in cryobox – separate boxes for swabs 1 and 2
	1.2	Cryobox transferred to Esky to keep out of sun
	1.3	Blood spots stored on pencils in polystyrene box
	1.4	At the end of each sample collection day, the team should return to base clinic
	1.5	Swabs to be transferred directly to the fridge [NB. If the fridge will not fit more than one set of swabs, swab 1 should take priority]
	1.6	Blood spots to be further aired in the evening
	1.7	Blood spots to be transferred to individual ziplock bags and refridgerated
2	2.1	Team to sleep in clinic or related housing where possible
	2.2	Following one week of sample collection, all swabs and blood spots to be returned to Lata for freezing. [NB. Ice packs should be used in transit where possible]
	2.3	Upon return to Lata, first swabs transferred immediately to portable -80 freezer.
	2.4	Upon return to Lata blood spots to be transferred immediately to Lab freezer
	2.5	Upon return to Lata, second swabs to be transferred to freezer if there is

		space, to refridgerator if no space at -20, or under bench next to portable -80 if not.
	2.6	Upon amalgamation of samples in Lata, samples to be returned to Honiara for storage directly in -20 freezer in NRH.
	2.7	Samples to be collected from NRH for transit to London.

Standard Operating Procedure: Conjunctival swabs

v.070916 – Robert Butcher

Introduction

This protocol should be used for the collection of conjunctival swabs from people of any age for diagnosis of conjunctival infection with *Chlamydia trachomatis*. This protocol is taken from Solomon et al. (Solomon et al., 2003) and validated for use in ddPCR diagnostics by Roberts et al. (Roberts et al., 2013).

Staff and Materials

Nurse (for everting eyelid and specimen collection)
Data manager (for data and swab collection, swab labeling and sample management)
Swabs (1 box = 100 swabs)
Polystyrene tubes (1 bag = 50 tubes)
Labels (1 page = 17 labels)
Cryobox (1 box = 100 places)
Reward (Coconut biscuits, banana, small taiyo etc.)

Standard Operating Procedure

1. Ensure participant consents to take part – this should be them (participant 18+ years) or their guardian (participant <18 years) filling in a paper consent form and signing.

2. Seat the participant in front of the grader.

3. Grader wears gloves for examination.

4. Grader everts and grades right eyelid.

5. Data Manager records clinical data.

6. Data manager prepares swab for Grader

7. Grader takes swab, taking care not to touch the head to avoid contamination, and passes it three times across the everted conjunctiva.

8. Data manager prepares swab collection tube by labeling with study ID.

9. Data manager receives swab from grader and closes tube.

10. Grader examines and records clinical presentation of left eye.

11. Data manager places swab in cryobox.

12. Cryobox should be transferred to refrigerator within 24 hours. Swabs can be stored at +4°C. After 1 week the swabs should be transferred to a freezer for storage at -20°C.

SOP 7.03
Droplet Digital PCR

Chlamydia research programme

Version History

V.7.01	-	13th July, 2012	Chrissy h. Roberts
V.7.02	-	13th July, 2012	Chrissy h. Roberts
V.7.03	-	30th July 2014	Robert Butcher

Summary

This protocol describes a method for detection and quantitation of *Chlamydia trachomatis* using the Bio-rad QX100 droplet digital PCR platform. The protocol was originally developed by Chrissy h. Roberts (Roberts *et al.*, *J. Clin. Micro.* 2013) and Anna Last (Last *et al.*, *J. Clin. Micro.* 2014), and subsequently updated based on ongoing optimisation.

Molecular targets

ENDOGENOUS CONTROL: *Homo sapiens* ribonuclease P/MRP 30kDa subunit (gi|13937783|gb|BC006991.1|)

*Forward (Hs-RPP30-F)	5' AGA TTT GGA CCT GCG AGC G 3'
*Reverse (Hs-RPP30-R)	5' GAG CGG CTG TCT CCA CAA GT 3'
^Probe (Hs-RPP30_HEX_BHQ1)	5' [HEX] TTC TGA CCT GAA GGC TCT GCG CG [BHQ1] 3'

Amplicon sequence, primer/probe binding sites:

```
agatttggacctgcgagcgggttctgacctgaaggctctgcgcggacttgtggagacagccgctc
******************  ^^^^^^^^^^^^^^^^^^^^^^^^ ********************
```

TARGET ONE: *Chlamydia trachomatis* cryptic plasmid ORF2 (pLGV440, X06707; SV 3; circular; genomic DNA; STD; PRO; 7500 BP.)

*Forward (Ct-Plasmid-F)	5' CAG CTT GTA GTC CTG CTT GAG AGA 3'
*Reverse (Ct-Plasmid-R)	5' CAA GAG TAC ATC GGT CAA CGA AGA 3'
^Probe (Ct-plasmid-PROBE-QUENCHER)	5' [PROBE] CCC CAC CAT TTT TCC GGA GCG A [QUENCHER] 3'

Amplicon sequence, primer/probe binding sites:

```
cagcttgtagtcctgcttgagagaacgtgcgggcgatttgccttaaccccaccatttttccggagcgagttacgaagacaaaacctctt
**********************                    ^^^^^^^^^^^^^^^^^^^^^^^^                 ****

cgttgaccgatgtactcttg
********************
```

TARGET TWO: *Chlamydia trachomatis OmcB* (serovar A).

*Forward (Ct-OMCB-F)	5' GAC ACC AAA GCG AAA GAC AAC AC 3'
*Reverse (Ct-OMCB-R)	5' ACT CAT GAA CCG GAG CAA CCT 3'
^Probe (Ct-OMCB-PROBE-QUENCHER)	5' [PROBE] CCA CAG CCA AAG AGA CTC CCG TAG ACC G [QUENCHER] 3'

Amplicon sequence, primer/probe binding sites:

```
gacaccaaagcgaaagacaacacttctcataaaagcaaaaaagcaagaaaaaaccacagcaaagagactcccgtagaccgtaaagaggt
************************                          ^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^     ****
tgctccggttcatgagt
*****************
```

Materials and reagents

Italics denote currently used suppliers. Approximate amounts required shown in brackets. As of September 2014, the total cost excluding DNA extraction was approximately GB£3.50 per well.

Reagent	Catalogue number
Bio-rad	
Bio-rad ddPCR supermix for probes (1.1 mL required per 96-well plate)	186-3027
Bio-rad ddPCR Droplet Generator Cartridges and Gaskets (1 cartridge and 1 gasket per plate column)	186-4007
Bio-rad ddPCR Droplet Generator Oil (7 mL per 96-well plate)	186-3005
Bio-rad ddPCR Droplet Reader Oil (1 L per 6 plates)	186-3004
Fisher Scientific	
Fisher Easy pierce foil seals (one per plate)	11979355
Eppendorf twin.tec 96-well semi-skirted plate (92 samples plus 4 control wells per plate)	10464393
Life Technologies MicroAmp clear adhesive film (one per plate)	10595025
Fisher sterile 2.0 mL tube	11568322
Fisher molecular biology-grade water	10505854
Fisher Tris-EDTA (1X)	10021703
Wolf Laboratories	
Sterile filter tips (1000 µL)	BT1250
Sterile filter tips (200 µL)	BT200
Sterile filter tips (20 µL)	BT20
Sigma Aldrich	
Primers and Probes (as above)	N/A

Preparation of primers and probes

Primer and probe sequences taken from Last *et al. J. Clin. Microbiol.* (2014). 52(1): 324-327.

ALL STOCK SOLUTIONS should be at 100 µM.

Primer/probe volumes for smaller well numbers can be calculated using $C_1 \times V_1 = C_2 \times V_2$

Diagnostic screen (*C. trachomatis* plasmid, *H. sapiens* RPP30)

9 µM Human RPP30 and *C. trachomatis* plasmid primers
2 µM Human RPP30 and *C. trachomatis* plasmid probes

NOTE: THIS CAN BE SCALED FOR LARGER VOLUMES

To prepare 230 µL (enough for one 96-well plate plus 10 µL excess)

	volume
100 µM Hs-RPP30-F	20.7 µL
100 µM Hs-RPP30-R	20.7 µL
100 µM Hs-RPP30-HEX-BHQ1	4.6 µL
100 µM Ct-Plasmid-F	20.7 µL
100 µM Ct-Plasmid-R	20.7 µL
100 µM Ct-Plasmid-FAM-BHQ1	4.6 µL
0.1X TE buffer (sterile/nuclease free)	138 µL
total	230 µL

Quantitative assay (*C. trachomatis OmcB, C. trachomatis* plasmid)

NOTE: THIS CAN BE SCALED FOR LARGER VOLUMES

3 µM *C. trachomatis* plasmid and *OmcB* primers
3 µM *C. trachomatis* plasmid and *OmcB* probes

To prepare 230 µL (enough for one 96-well plate plus 10 µL excess)

	volume
100 µM Ct-OMCB-F	6.9 µL
100 µM Ct-OMCB-R	6.9 µL
100 µM Ct-OMCB-FAM-BHQ1	6.9 µL
100 µM Ct-Plasmid-F	6.9 µL
100 µM Ct-Plasmid-R	6.9 µL
100 µM Ct-Plasmid-HEX-BHQ1	6.9 µL
0.1X TE buffer (sterile/nuclease free)	188.6 µL
total	230 µL

PCR setup

WORK IN A PCR CLEAN & DNA FREE ENVIRONMENT

Each assay uses EITHER diagnostic OR quantitative primer/probe mixes.

CRITICAL STEP: MIX AND INVERT THE SUPERMIX AND PCR MIXES EXTREMELY WELL, THEY ARE VISCOUS AND THE TESTS WILL FAIL IF YOU DON'T DO THIS PROPERLY.

Composition per reaction

	1X
2X ddPCR supermix	11 µL
Primer/probe supermix (10X each of six oligos)	2.2 µL
Sample DNA	8.8 µL
total	22 µL

001 I To prepare sufficient ddPCR MASTERMIX to run one 96 well plate (100 reactions)

In a 2 mL microcentrifuge tube, add the following:

	100X
2X ddPCR supermix	1100 µL
Primer/probe mix (10X each of six oligos)	220 µL
total	1320 µL

MOVE TO DNA LOADING/PCR CLEAN AREA

002 I To each well of a 96-well semi-skirted Eppendorf twin-tec plate (in retainer), aliquot 13.2 µL ddPCR mastermix. Use reverse pipetting to allow rapid distribution of viscous material.

003 I Aliquot 8.8 µL of DNA from samples to each well of the plate. Wells 12E and 12F should be taken up by water samples as negative controls. Wells 12G and 12H should be taken up by known *C. trachomatis* and *RPP30* positive samples (synthetic) as positive PCR controls.

004 I Seal with microamp cover film (ensure close seal) and vortex gently (1000 rpm) to mix reagents for 30 seconds

005 I Centrifugate at 1000 rcf for 1 minute to pool PCR mix at bottom of plate.

006 I Proceed to droplet generation

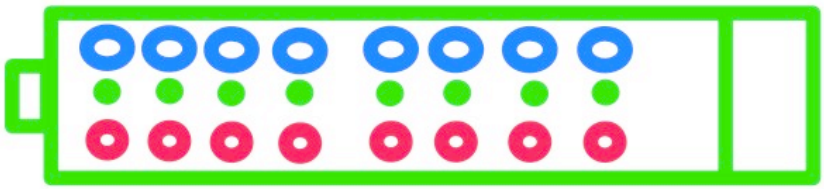
Droplet generation

TURN ON THE HEAT SEALER

007 I Place a new, clean and Eppendorf twin-tec semi-skirted 96-well plate in a retainer

008 I Carefully remove the microamp film, without disturbing the PCR mixes

009 I Place a new droplet generation cartridge in the droplet cassette, the notch should face left (SEE FIGURE). Close the cassette by applying gentle pressure to the short edges.



010 I Transfer 70 µL droplet generation oil to each oil well of the cartridge (red on figure, marked "oil" on the cassette")

011 I Transfer 20 µL of each PCR mixture from row 1 in to each sample well of the cartridge (green on figure, marked "sample" on the cassette). Take care to avoid introducing air by putting pipette tips to the lowest point where the wall of the vessel meets the base. Hold pipette at around 20 degree angle to wall, actuate slowly and raise tips from the base as the wells continue to fill

012 I Attach a rubber gasket to the notched teeth on the cassette. Take care not to move the rubber across the wells as this could lead to cross-contamination. Ensure that the four teeth are properly engaged with the gasket as failure to do this will prevent a good seal forming.

013 I Transfer the cassette to the droplet generation machine. Holding the cassette gently at the top and bottom, place on the magnetised platform. A green light will indicate proper engagement.

014 I Activate droplet generation by pressing the button.

015 I Prepare the next cassette.

016 I When the first droplets are complete, exchange the first cassette for the second in the droplet generator. Reactivate the device.

017 I Carefully aspirate 45-50 µL of droplets from the droplet wells. Wait for 10s for droplets to float to the top of the pipette tip, then carefully expel excess oil back into the cartridge, taking care not to disturb the droplet layer. Ensure that aspiration is performed slowly (around 10 s) as failure to do this will lead to droplet instability.

018 I Carefully expel the remaining oil and droplets (blue on figure) to row 1 of the droplet plate. Ensure that expulsion is performed slowly (around 10 s) as failure to do this will lead to droplet instability.

019 I Use a short length of lab tape to temporarily seal the wells of row 1.

020 I Prepare the droplet cassette for row 3, then transfer the droplets from row 2. Continue this process until the plate is completed.

021 I Remove all lab tape and replace with an easy-pierce heat seal, ensuring that it is the right way up.

022 I Seal the tray for 3 seconds at 170 degrees (ddPCR protocol). Release, turn the plate through 180 degrees, then repeat sealing for another 3 seconds.

023 I Put plate in Applied Biosystems thermal cycler and run the program "DDPCR". Thermal cycling parameters are:

10 minutes at 95°C (activation), 40 cycles of 15 seconds at 95°C (dissociation) followed by 1 minute at 60°C (annealing and extension phases combined to optimise exonuclease activity of DNA polymerase), 10 minutes at 98°C (deactivation), hold at 12°C.

024 I When the cycle is complete, droplets can be stored at 4°C for up to 48 hours, or counted immediately.

DROPLET READING

If this is the first read of the week, start by performing a prime/flush/prime operation. The prime operation must be completed before each run.

025 l Transfer the plate to the special retainer and place this device in the droplet reader. A green light will indicate correct engagement with the platform. Close the door by pressing the button.

026 l Open the QuantaSoft software and create a new template.

027 l Select all wells on the plate and double click on the plate diagram to edit the details.

028 l For sample names, all wells should be labelled appropriately. Channel one detects FAM and channel two detects VIC or HEX. These should be labelled with the appropriate targets in the PCR mix.

029 l Start the droplet count by pressing the run button

ANALYSIS

030 l After the run has finished, check the positive and negative controls to ensure (a) the assay has worked and (b) there is no systematic contamination of PCR reagents. Using the positive and negative controls as a guide, manually set a threshold for each well. The threshold should be set as close as possible to the negative population, as PCR inhibition causes reduced fluorescence amplitude of positive droplets.

031 l After gating, the data table should be exported as a .csv filetype.

032 l Create a new folder for ddPCR data containing a subfolder entitled "out". Save the exported data in the ddPCR data folder.

033 l Data should be processed using free R software and the script in Appendix A which generates concentration estimates for both channels, and calls positives and negatives based on a 95% confidence in non-zero load, as described by Roberts *et al.* Following analysis, remove output data files from the "out" folder to avoid overwriting them with subsequent analyses.

Appendix A

```
##############################################################################################
#                          Open source license                                               #
##############################################################################################
# To attribute this work, you must cite the name of the original source, the name of        #
# the author (Chrissy h. Roberts) and their contact details                                 #
# (chrissyhroberts@yahoo.co.uk). This work is licensed under the Creative Commons           #
# Attribution-ShareAlike 3.0 Unported License.                                              #
# To view a copy of this license, visit http://creativecommons.org/licenses/by-sa/3.0/      #
# or send a letter to Creative Commons, 444 Castro Street, Suite 900, Mountain View,        #
# California, 94041, USA.                                                                   #
##############################################################################################

delete.all <- function()
  rm(list=ls(pos=.GlobalEnv), pos=.GlobalEnv)
delete.all()
ls()
setwd(dir="C:\\Users/Bertie/Dropbox/Bert/ddPCRv1/")
cwd <-getwd()
cwd
cat(cwd,"/out/",sep="")
outwd <-paste(cwd,"/out/",sep="")
path <- cwd
outpath <- outwd

#READ IN PLATE
samples=read.delim(file.path(path,"140616_cov_final.csv"),header=T,sep=",",na.strings="NA")
View(samples)
samples$famposvicpos<-samples$Ch1.Ch2.
samples$Ch1.Ch2.<-NULL
samples$famposvicneg<-samples$Ch1.Ch2..1
samples$Ch1.Ch2..1<-NULL
samples$famnegvicpos<-samples$Ch1.Ch2..2
samples$Ch1.Ch2..2<-NULL
samples$famnegvicneg<-samples$Ch1.Ch2..3
samples$Ch1.Ch2..3<-NULL
samples$Freq<-(samples$famposvicneg+samples$famnegvicpos+samples$famposvicpos+samples$famnegvicneg)
View(samples)
#calculate values for poisson calculation.
#phat is estimator of P, the probability of a micelle being positive. P is unknown but phat can be estimated by phat =
number of positive droplets / total droplets
samples$phat_fam<-((samples$famposvicneg+samples$famposvicpos)/samples$Freq)
samples$phat_vic<-((samples$famnegvicpos+samples$famposvicpos)/samples$Freq)

#standard deviation of p_hat estimator
samples$sd_phat_fam<- sqrt((samples$phat_fam*(1-samples$phat_fam))/samples$Freq)
samples$sd_phat_vic<- sqrt((samples$phat_vic*(1-samples$phat_vic))/samples$Freq)

#upper and lower confidence intervals (95%) of p_hat estimates
samples$phat_low_fam <- samples$phat_fam - (1.96 * samples$sd_phat_fam)
samples$phat_high_fam <- samples$phat_fam + (1.96 * samples$sd_phat_fam)

samples$phat_low_vic <- samples$phat_vic - (1.96 * samples$sd_phat_vic)
samples$phat_high_vic <- samples$phat_vic + (1.96 * samples$sd_phat_vic)

#lambda is the true concentration of target molecules per chamber. It is unknown but can be estimated by lambda hat.
lambda_hat =  -ln (1-p_hat)

samples$lambda_hat_fam<-(-log(1-samples$phat_fam))
samples$lambda_hat_vic<-(-log(1-samples$phat_vic))

#upper and lower confidence intervals for lambda hat :
samples$lambda_hat_low_fam <- (-log(1-samples$phat_low_fam))
samples$lambda_hat_high_fam <- (-log(1-samples$phat_high_fam))

samples$lambda_hat_low_vic <- (-log(1-samples$phat_low_vic))
```

```
samples$lambda_hat_high_vic <- (-log(1-samples$phat_high_vic))

# lambda_hat = concentration target per micelle
# lambda_hat_low = lower 95% CI for lambda hat
# lambda_hat_high = upper 95% CI for lambda hat

# calculate concentration of target molecules per reaction.

samples$fam_copies_per_reaction<-samples$lambda_hat_fam*samples$Freq
samples$vic_copies_per_reaction<-samples$lambda_hat_vic*samples$Freq

samples$fam_copies_per_reaction_low<-samples$lambda_hat_low_fam*samples$Freq
samples$fam_copies_per_reaction_high<-samples$lambda_hat_high_fam*samples$Freq

samples$vic_copies_per_reaction_low<-samples$lambda_hat_low_vic*samples$Freq
samples$vic_copies_per_reaction_high<-samples$lambda_hat_high_vic*samples$Freq

samples$volume<-samples$Freq*0.91e-3
samples$concentration_fam_average_copies_uL <- samples$fam_copies_per_reaction/samples$volume
samples$concentration_fam_low_copies_uL <- samples$fam_copies_per_reaction_low/samples$volume
samples$concentration_fam_high_copies_uL <- samples$fam_copies_per_reaction_high/samples$volume

samples$concentration_vic_average_copies_uL <- samples$vic_copies_per_reaction/samples$volume
samples$concentration_vic_low_copies_uL <- samples$vic_copies_per_reaction_low/samples$volume
samples$concentration_vic_high_copies_uL <- samples$vic_copies_per_reaction_high/samples$volume
rownumbers<-c(1:length(samples$Well))
for (i in
rownumbers){if(is.na(samples$concentration_fam_high_copies_uL[i])){samples$concentration_fam_high_copies_uL[i]<-
samples$concentration_fam_average_copies_uL[i]}else{next}}
for (i in
rownumbers){if(is.na(samples$concentration_vic_high_copies_uL[i])){samples$concentration_vic_high_copies_uL[i]<-
samples$concentration_vic_average_copies_uL[i]}else{next}}

for (i in
rownumbers){if(is.infinite(samples$concentration_fam_average_copies_uL[i])){samples$concentration_fam_average_copi
es_uL[i]<-20000}}
for (i in
rownumbers){if(is.infinite(samples$concentration_fam_low_copies_uL[i])){samples$concentration_fam_low_copies_uL[i]<
-20000}}
for (i in
rownumbers){if(is.infinite(samples$concentration_fam_high_copies_uL[i])){samples$concentration_fam_high_copies_uL[i]
<-20000}}
for(i in
rownumbers){if(is.infinite(samples$concentration_vic_average_copies_uL[i])){samples$concentration_vic_average_copies
_uL[i]<-20000}}
for(i in
rownumbers){if(is.infinite(samples$concentration_vic_low_copies_uL[i])){samples$concentration_vic_low_copies_uL[i]<-
20000}}
for(i in
rownumbers){if(is.infinite(samples$concentration_vic_high_copies_uL[i])){samples$concentration_vic_high_copies_uL[i]<-
20000}}

#plot graphs of calculated concenentration (copies/uL) values with error bars
#library(gplots)

pdf(file.path(outpath,"001_concentration_vic.pdf"))
plot (samples$concentration_vic_average_copies_uL,pch=46,cex=4,log="y",xlab="Sample",ylab="HURNASE
(copies/uL)",xaxt="n",cex.axis=0.4,xlim=c(0,100),ylim=c(0.1,(max(samples$concentration_vic_high_copies_uL)*1.1)))
for(i in
rownumbers){arrows(i,samples$concentration_vic_average_copies_uL[i],i,samples$concentration_vic_high_copies_uL[i],l
ength=0.02,angle=90,code=2,col="red")}
for(i in
rownumbers){arrows(i,samples$concentration_vic_average_copies_uL[i],i,samples$concentration_vic_low_copies_uL[i],le
ngth=0.02,angle=90,code=2,col="red")}
axis(1,rownumbers,labels=samples$Var1,cex.axis=0.4)
grid()
dev.off()
```

```
pdf(file.path(outpath,"001_concentration_fam.pdf"))
plot (samples$concentration_fam_average_copies_uL,pch=46,cex=4,log="y",xlab="Sample",ylab="C.trachomatis Plasmid
(copies/uL)",xaxt="n",cex.axis=0.4,xlim=c(0,100),ylim=c(0.1,(max(samples$concentration_fam_high_copies_uL)*1.1)))
for(i in
rownumbers){arrows(i,samples$concentration_fam_average_copies_uL[i],i,samples$concentration_fam_high_copies_uL[i
],length=0.02,angle=90,code=2,col="red")}
for(i in
rownumbers){arrows(i,samples$concentration_fam_average_copies_uL[i],i,samples$concentration_fam_low_copies_uL[i]
,length=0.02,angle=90,code=2,col="red")}
axis(1,rownumbers,labels=samples$Var1,cex.axis=0.4)
grid()
dev.off()
View(samples)
samples$FAILS<-NA

#DEFINE FAILS AND RESULTS
for (i in rownumbers) {if (samples$concentration_fam_low_copies_uL[i]<0){samples$FAILS[i]<-"WARNING : CI for FAM
crosses zero"}}
for (i in rownumbers) {if (samples$famnegvicneg[i]<(0.01*samples$Freq[i])){samples$FAILS[i]<-"WARNING: REACTION
MAY BE SATURATED WITH TOO MANY TEMPLATES"}}
for (i in rownumbers) {if (samples$concentration_vic_low_copies_uL[i]<0.0000001){samples$FAILS[i]<-"FAIL: HUMAN
DNA CONCENTRATION TOO LOW"}}

#define amount of area under the curve that is above zero
for (i in rownumbers){samples$areaabovezerofam[i]<-1-
pnorm(0,mean=samples$phat_fam[i],sd=samples$sd_phat_fam[i])}
for (i in rownumbers){samples$areaabovezerovic[i]<-1-pnorm(0,mean=samples$phat_vic[i],sd=samples$sd_phat_vic[i])}

#assign endogenous control status
samples$endogenouscontrolresult<-NA
for (i in rownumbers) {if (samples$areaabovezerovic[i]>=0.95){samples$endogenouscontrolresult[i]<-"Endogenous control
OK"}}
for (i in rownumbers) {if (samples$areaabovezerovic[i]<0.95){samples$endogenouscontrolresult[i]<-"Endogenous control
failed"}}

#assign warnings based on FI average values and 95% CI platewide
samples$targetresult<-NA
for (i in rownumbers) {if (samples$areaabovezerofam[i]>=0.95){samples$targetresult[i]<-"Sample is positive for the
target"}}
for (i in rownumbers) {if (samples$areaabovezerofam[i]<0.95){samples$targetresult[i]<-"Sample is negative for the
target"}}

#print output
write.table(samples, file = (file.path(outpath,"000_results_full.txt")),row.names=F,col.names=T,quote=F,sep="\t")
columns<-c(1,55,56,47,48,49,50,51,52,53,54,2,13,14,15,16,11,12,46)
write.table(samples[,columns], file =
(file.path(outpath,"000_results_concise.txt")),row.names=F,col.names=T,quote=F,sep="\t")
View(samples)

##############################################################################################
#END OF SCRIPT dropletR_man_thresholds.R
##############################################################################################

#extract columns required for desired output and print summary table
#samples_summary<-
subset(samples,select=c("Sample","Ratio","concentration_fam_average_copies_uL","concentration_vic_average_copies_
uL"))
#write.table(samples_summary,file = (file.path(outpath,"micro.txt")),row.names=F,col.names=T,quote=F,sep="\t")
```

ELISA for detection of antibodies against *C trachomatis* antigen pgp3
V4.6 – Summer 2015 beta testing

Solutions and Reagents

Included:

Controls: (human sera with known ratios of pgp3 antibodies)

- Aliquot to 30µl per tube
- Store long term at -80°C. Working aliquot may be stored at -20°C
- Thaw on ice or at 4°C-10°C (Minimize freeze-thaw: no more than 5x/aliquot)
- Dilute 1:50 in PBST-milk [2.5µl + 125µl PBST-milk per plate]

Antigen: (recombinant GST tagged pgp3 in PBS, 0.25mg/ml)

- Aliquot to 120µl per tube
- Store long term at -80°C. Working aliquot may be stored at -20°C
- Thaw on ice or at 4°C-10°C (Minimize freeze-thaw: no more than 5x/aliquot)
- **Do not vortex**. Tap gently or pipette up and down to mix.
- Dilute 1:500 in Sensitizing Buffer [11µl + 5.5mL Sensitizing Buffer per plate]

Secondary Antibody: (mouse anti-human IgG(Fc)-HRP, Southern Biotech, cat no 9042-05)

- Store at 4°C-10°C
- Dilute 1:10,000 in PBST [0.55 µl + 5.5mL PBST per plate]

Substrate: TMB (SureBlue, 1 component, KPL, cat no 52-00-00)

- Store at 4°C-10°C
- Ready to use, must be room temperature for assay.

Not Included:

Sensitizing Buffer: (0.1M $NaHCO_3$, pH 9.6)

- For coating the plate with pgp3. One liter can coat up to 200 plates.
- 8.40 g $NaHCO_3$ qs to 1 liter with dH_2O, pH with NaOH to achieve pH 9.6
- Vacuum filter solution. Store at 4°C.

PBST: (1x PBS pH 7.2 (ion free) + 0.3% Tween-20)

- For blocking plate, washing plate, and diluting antibody
- 1000 ml PBS + 3 ml Tween-20
- Stir gently for 15 minutes

PBST-milk: (0.3% PBS/T + 5% nonfat milk powder)

- For eluting/diluting blood spots; make fresh daily
- 50mL PBST + 2.5g milk powder
- Stir gently for 15 minutes

Stop Solution: 1N H_2SO_4

- For stopping TMB/HRP reaction
- 28mL 96% (18M) H_2SO_4 + 472mL dH_2O

  Caution: H_2SO_4 is extremely corrosive – use concentrated form only in fume hood

Equipment:

Plate reader
Analysis software (Excel)
Plate shaker (orbital), room temperature
Multichannel pipette, P1000, P200, P20, P2 single channel pipettes+ tips

Consumables

Plates: (Immulon 2HB, ThermoScientific cat# 3455)

- Assay plate.
- Can run 42 samples/plate

Lids/sealing film for plates [covering plates during incubation steps]
Microcentrifuge tubes [sample dilution]
Round bottom 96 well titer plates [for preload plate]
Paper towels, bleach [wash steps, waste disposal]
Multichannel reservoirs [pipetting]
50mL and 15mL conical tubes [reagent dilution]

Before Starting

Plate Washing:

Choose method that is practical in lab in which the assay will be run

- Pipette: load 200µl/well with a multichannel. Do not touch bottom of well. Decant into sink to remove.
- Automatic plate washer: 200µl/well
- Squirt bottle not recommended due to higher CVs.

- Between washes, tap plates 4-8 times firmly on a pile of paper towels to remove excess liquid.
    - paper towels can be reused for several ELISAs

Examine plates for bubbles. These can be gently popped using air from an empty squeeze bottle, a fine gauge needle or pipette tip. It is very important no large bubbles remain prior to the next step. Be careful not to scratch the bottom of the plate when popping bubbles.

Adding samples to well:

If using the multichannel is awkward, a regular p200 pipet can be used instead. In this case, a preload plate is not necessary. A multichannel is necessary to add TMB and Stopping Solution due to the speed of the reaction.

Suggested plate layout:

	1	2	3	4	5	6	7	8	9	10	11	12
A	1000u	1000u	U03	U03	U11	U11	U19	U19	U27	U27	U35	U35
B	500u	500u	U04	U04	U12	U12	U20	U20	U28	U28	U36	U36
C	200u	200u	U05	U05	U13	U13	U21	U21	U29	U29	U37	U37
D	50u	50u	U06	U06	U14	U14	U22	U22	U30	U30	U38	U38
E	NHS	NHS	U07	U07	U15	U15	U23	U23	U31	U31	U39	U39
F	BLANK	BLANK	U08	U08	U16	U16	U24	U24	U32	U32	U40	U40
G	U01	U01	U09	U09	U17	U17	U25	U25	U33	U33	U41	U41
H	U02	U02	U10	U10	U18	U18	U26	U26	U34	U34	U42	U42

All samples and controls are run in duplicate (U01-U42)

Blank is PBST-milk alone

NHS = normal (negative) human serum

Before study samples are tested:

1. TMB optimization:

1. Evaluate 12 TMB incubation times on one plate with specified controls:
    a. TMB incubation times: Ranging from 4-16 min, increasing in one minute increments
    b. Controls: 1000, 200, NHS and blank
2. Stagger TMB addition to each column so H_2SO_4 addition occurs simultaneously (i.e. add TMB to last column first, then move backwards across plate: Column 12, then 11, then 10, etc.)

Suggested plate layout:

4 min	5 min	6 min	7 min	8 min	9 min	10 min	11 min	12 min	13 min	14 min	15 min
1000											
1000											
200											
200											
NHS											
NHS											
BLANK											
BLANK											

Data analysis:

Optimal TMB incubation should be chosen by the following criteria:

1. The 200 normalization standard should be around **1 OD (0.8-1.2 OD is acceptable)**
2. The range between the 1000 control and NHS should be > **1.5 OD**
3. The ratio between the 1000 control and NHS should be > **10**
4. If several consecutive time points meet this criteria, choose a time point in the middle of this range

Example:

	4 min	5 min	6 min	7 min	8 min	9 min	10 min	11 min	12 min	13 min	14 min	15 min
1000	1.399	1.532	1.736	1.843	1.927	2.019	2.096	2.095	2.190	2.211	2.294	2.272
200	0.545	0.690	0.678	0.849	0.982	1.009	1.096	1.138	1.215	1.277	1.332	1.294
NHS	0.089	0.098	0.138	0.145	0.179	0.184	0.194	0.199	0.204	0.217	0.232	0.237
Signal:Noise	15.7	15.7	12.6	12.7	10.7	11.0	10.8	10.5	10.8	10.2	9.9	9.6
Range	1.3	1.4	1.6	1.7	1.7	1.8	1.9	1.9	2.0	2.0	2.1	2.0

2. Calculation of baseline values for controls

1. Run **10 plates** on at least **5 different days** to set baseline acceptable parameters for controls. **Do not start running clinical samples until baseline parameters have been established.**
    a. Run plates with **exact** conditions that will be used to run samples
    b. Normalize blanked OD_{450} values to the blanked OD_{450} of the 200u control.
    c. Add and subtract 20% to the average to calculate the upper and lower limit for controls
    d. Take ratio of 1000u to 0u (i.e. NHS) to calculate signal: noise ratio. Should be average of 10.
    e. Subtract the OD value of 0u from 1000u to calculate range. Should be greater than 1.5.

Example:

1	2	3	4	5	6	7	8	9	10	11	12
1000											
500											
200											
50											
NHS											
BLANK											

Raw OD	14-Apr 1	15-Apr 2	16-Apr 3	16-Apr 4	17-Apr 5	22-Apr 6	23-Apr 7	23-Apr 8	24-Apr 9	24-Apr 10	Average	Percent CV
1000	1.98	1.94	2.13	1.80	1.63	1.85	1.65	1.66	1.78	1.51	1.79	10.5
500	1.56	1.54	1.71	1.49	1.33	1.51	1.15	1.28	1.32	1.09	1.40	13.9
200	1.03	1.09	1.25	1.03	0.92	0.95	0.77	0.83	0.87	0.78	0.95	15.9
50	0.42	0.44	0.58	0.53	0.43	0.35	0.35	0.40	0.35	0.37	0.42	18.7
0	0.17	0.16	0.27	0.23	0.21	0.13	0.15	0.20	0.17	0.14	0.18	23.6
Signal:Noise	12	12	8	8	8	14	11	8	11	11	10	
Range	1.8	1.8	1.9	1.6	1.4	1.7	1.5	1.5	1.6	1.4	1.6	

200 norm	14-Apr 1	15-Apr 2	16-Apr 3	16-Apr 4	17-Apr 5	22-Apr 6	23-Apr 7	23-Apr 8	24-Apr 9	24-Apr 10	Average	Percent CV	Upper Limit	Lower Limit
1000	1.91	1.78	1.70	1.75	1.77	1.95	2.14	2.00	2.05	1.93	1.90	7.6	2.28	1.52
500	1.50	1.41	1.36	1.45	1.45	1.59	1.49	1.55	1.53	1.40	1.47	4.8	1.77	1.18
50	0.40	0.40	0.46	0.52	0.46	0.37	0.45	0.49	0.40	0.47	0.44	10.5	0.53	0.36
0	0.17	0.15	0.21	0.22	0.23	0.14	0.20	0.24	0.19	0.18	0.19	18.5	0.23	0.15

ELISA procedure: one day prior to testing:

1. Dilute pgp3 to 500ng/mL in sensitizing buffer to make antigen coating solution
    - 11µl of 0.25mg/mL pgp3 + 5.5mL sensitizing buffer [per plate]
2. Add 50µl antigen coating solution per well (25ng/well) and shake on plate shaker briefly to ensure that wells are evenly coated. Place in sealed Ziploc bag with folded wet paper towel overnight at 4°C-10°C.
3. Dilute all samples 1:50 in PBST-milk and keep overnight at 4°C – 10°C.
    - 1 dried blood spot + 250µl PBST-milk
    - 2.5µl sera + 125µl PBST-milk (includes all standards and controls)

ELISA procedure: day of assay

***make PBST fresh weekly**
***get TMB out of 4°C at the beginning of the day to come to room temperature**
***Do not reuse sealing films, use a new one for each step**

1. Decant antigen and wash 2x with PBST. Add 100µl PBST to each well to block plate for **1 hour** at 4°-10°C
    - Plate can be rocking or stationary

2. During blocking step:
    - Remove samples from refrigerator and briefly vortex to ensure a homogeneous solution. All samples must be at room temperature (RT) before step 4.
    - If using a multichannel to load plate, create preload plate by adding 130µl each sample per well to a round bottom 96 well plate. This will be enough for 1 set of duplicates.

3. Decant blocking solution. Tap firmly 2x to remove excess liquid. No wash step is necessary.

4. Mix samples by pipetting up and down and add 50µl of each sample per well by reverse pipetting. Run controls and samples in duplicate.

5. Incubate plate at RT on orbital shaker for **2 hours**.

6. Remove samples by decanting into sink with 10% bleach--avoid splash back. Wash 4x with PBST.

7. Dilute secondary antibody 1:10,000 in PBST.
    - 0.55 µl anti-human-IgG-HRP + 5.5mL PBST [per plate]

8. Add 50µl secondary antibody to each well by reverse pipetting and incubate at RT on shaker for **1 hour**.

9. Wash 4x with PBST.

10. Add 50µl TMB to each well. Incubate at RT protected from light, no shaking

11. Add 50µl 1N H_2SO_4 per well to stop the reaction (same order as TMB was added). Shake briefly to mix.
    - **It's very important that no bubbles remain prior to reading the plate**

12. Read plate **immediately** at 450nm.

Analysis procedure

1. Per plate quality control

Internal Controls

- Dynamic range of ≥ 5 is acceptable.
- CVs for control samples <15% is acceptable
- Normalized values fall within acceptable parameters
    i. Repeat plate if both 3/4 controls are outside range

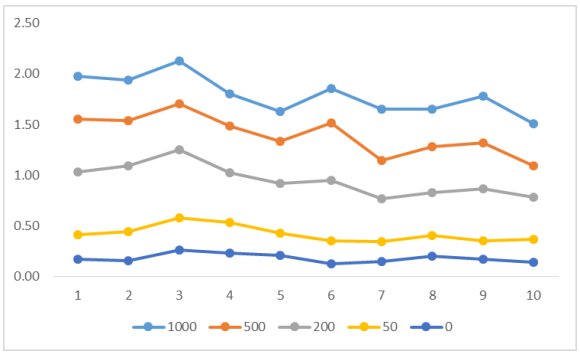
Duplicate values of samples

- %CV between OD_{450} of duplicates should be <15%.
    i. If both duplicates are 20% below the cutoff, then sample does not need to be rerun

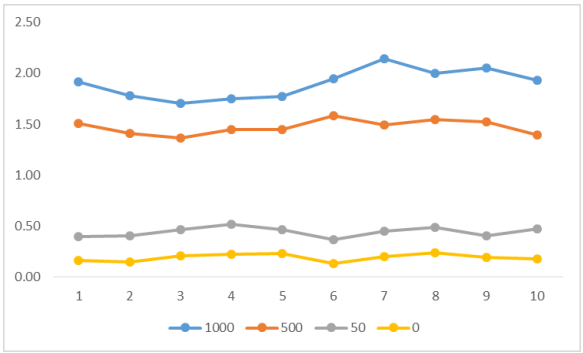
2. Weekly, ongoing/cumulative quality control

- Record all OD and normalized values from standard curve and controls in a single graph or table. Observe trend weekly to make sure no component of the ELISA is going bad and skewing results.

Blanked OD450



Normalized to 200u



i. If deviations from average of 10 plate baseline of >20% is seen on 3 consecutive plates, then do the following and run only standards until controls fall within normal parameters.
    1. Make fresh PBST and coating buffer
    2. Use a fresh antigen aliquot
    3. Use a fresh aliquot of all controls

ii. If this does not work
    1. Have ambient conditions changed?
        a. Redo TMB optimization with a fresh bottle of TMB
    2. Change secondary antibody – contact CDC for replacement

Best practices

Avoid touching the bottom plate.

Sera and Antigen quality control

Both sera (used in controls) and pgp3 antigen are susceptible to degradation with repeated freeze/thaw.

Take the following precautions to minimize effects of freeze/thaw

- Thaw samples at 4°-10°C or on ice. Do not leave thawed samples at 4°C-10°C longer than overnight.
- **Do not vortex the antigen**
- Freeze/thaw any single aliquot **no more than 5x**
    - Aliquot sera in 30µl batches [enough for ~10 plates, run 2 per day]
    - Aliquot pgp3 (0.25mg/mL) in 120µl batches [enough for ~10 plates, run 2 per day]
- Store all aliquots except the ones actively in use at -80°C; active aliquots may be stored at -20°C
- Keep aliquots stored in screw cap microcentrifuge tubes. If only snap cap tubes are available, Parafilm tubes to prevent evaporation
- Discard any aliquots that show evidence of being degraded and switch to a fresh aliquot
    - e.g. controls gives value outside typical range in 3 consecutive tests

Safety

Components of this assay are derived from biological materials, so take the following precautions

- Sera and eluted blood spots should be handled under a biosafety cabinet if possible, especially if there is a high risk for generation of aerosols (e.g. pipetting, centrifugation).
- Use universal lab precautions: glove, lab coat, eye protection and close toed shoes—take special caution when decanting samples in sink to avoid splash back
- Sera and liquid biological derivatives should be mixed with bleach before disposal (final concentration of bleach should be no less than 10%).
- Work space surfaces should be wiped down with 70% ethanol before and after working with ELISA reagents.
- All solid waste should be treated as biohazardous for disposal—segregate and autoclave before throwing away.

Experiment Worksheet

Study: COR NTD alternate indicators pgp3 ELISA **Date:** _____

Samples/Reason for test: _____

Plate Layout:

	1	2	3	4	5	6	7	8	9	10	11	12
A	1000u	1000u										
B	500u	500u										
C	200u	200u										
D	50u	50u										
E	NHS	NHS										
F	BLANK	BLANK										
G												
H												

	1	2	3	4	5	6	7	8	9	10	11	12
A	1000u	1000u										
B	500u	500u										
C	200u	200u										
D	50u	50u										
E	NHS	NHS										
F	BLANK	BLANK										
G												
H												

Procedural Notes:

Step	Reagent	µl/well	Incubation	Lot/Date Made/Notes
Antigen	pgp3-GST, 25ng/well	50µl/well	o/n at 4°C	
Wash	PBS + 0.3% Tween	200µl/well	2x	
Blocking	PBS + 0.3% Tween	100µl/well	1hr at 4°C	
Sample	DBS or sera, 1:50	50µl/well	2hr at RT	
Wash	PBS + 0.3% Tween	200µl/well	4x	
Antibody	anti-IgG-HRP, 1:10,000	50µl/well	1hr at RT	
Wash	PBS + 0.3% Tween	200µl/well	4x	
Substrate	TMB, 1C	50µl/well	RT, opt. time	
Stop Sol'n	1N H2SO4	50µl/well	read immed.	